A Practical Guide to High Resolution Melt Analysis Genotyping

Sean Taylor, Rachel Scott, Richard Kurtz, Carl Fisher, Viresh Patel, and Frank Bizouarn, Bio-Rad Laboratories, Inc., Hercules, CA 94547

Introduction

Classifying and understanding genetic variation between populations and individuals is an important aim in the field of genomics. Many common diseases (diabetes, cancer, osteoporosis, etc.) and clinically relevant phenotypic traits are elicited from the complex interaction between a subset of multiple gene products and environmental factors. The unique genetic profile of an individual confers susceptibility to a given trait or disease. Consequently, there is a rapidly growing interest in quantifiable methods for mutation screening in life science research. Such methods are used for detecting genes responsible for diverse traits in organisms from all biological kingdoms. Some notable examples include resistance to parasites or yield in plants (Dracatos et al. 2008, Alves et al. 2008), drug resistance in microorganisms (Zhao et al. 2010), and genetic susceptibility to cancer in humans (Lascorz et al. 2010).

High resolution melt (HRM) analysis is the quantitative analysis of the melt curve of a DNA fragment following amplification by PCR and can be considered the next-generation application of amplicon melting analysis. It requires a real-time PCR detection system with excellent thermal stability and sensitivity and HRM-dedicated software. The combination of improved qPCR instrumentation and saturating DNA binding dyes has permitted the identification of genetic variation in nucleic acid sequences by the controlled melting of a double-stranded PCR amplicon. New instrument calibration methods coupled with HRM-compatible software permit the rapid analysis of the resulting data sets and the discrimination of DNA sequences based on their composition, length, GC content, or strand complementarity (Garritano et al. 2009).

HRM experiments generate DNA melt curve profiles that are both specific and sensitive enough to distinguish nucleic acid species based on small sequence differences, enabling mutation scanning, methylation analysis, and genotyping (Garritano et al. 2009). For example, HRM can be used to detect single-base sequence variations or to discover unknown genetic mutations. It can also be used to quantitatively detect a small proportion of variant DNA in a background of wild-type sequence at sensitivities approaching 5% to study somatically acquired mutations or changes in methylation state (Malentacchi et al. 2009). Furthermore, HRM is a nondestructive method and subsequent characterization of the associated amplicon using gel electrophoresis or sequencing, for example, can be performed after melt analysis.

High Resolution Melt Analysis: An Accessible Technique to Screen Targeted SNPs in Multiple Samples

The completion of the human genome sequence (Lander et al. 2001, Venter et al. 2001) enabled the identification of millions of single nucleotide polymorphisms (SNPs) and the construction of a high-density haplotype map (International HapMap Consortium 2005, Frazer et al. 2007). These combined data have opened the door for both small- and large-scale genotyping of case versus control populations of any organism. The government-funded project dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) is a publicly accessible database of all catalogued SNPs in the genomes of a growing list of model organisms and has become an important resource in the design of SNP profiling experiments. The ongoing discovery and study of new SNPs will help unravel the complex combinations of genetic modifications contributing to important traits in health and disease.

Genetic mutations may comprise a single nucleotide change or multiple base changes, insertions, and/or deletions. The detection and genotyping of SNPs, representative of the smallest genetic change, highlights the sensitivity of HRM analysis. SNPs have been categorized into four classes as summarized in Table 1. The class IV SNPs are both the rarest and most difficult to identify because they differ by a single hydrogen bond, resulting in the smallest melt curve temperature shift compared to class I through III.

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%

In order to perform successful HRM experiments and reliably detect subtle and rare sequence variants, such as Class IV SNPs, careful experimental design and HRM-specific analysis tools are required.

Processing Melt Curve Data for High Resolution Melt Analysis

Melt curves are run routinely for qPCR experiments to ensure primer specificity, with data typically collected over a temperature range between 65 and 95ºC in 0.5ºC increments. For HRM experiments, data are generally collected at narrower temperature increments than for standard melt curve protocols, commonly in 0.2ºC increments. Depending on the particular template and experimental conditions, the increased density of data points collected can assist with melt profile generation and subsequent sequence discrimination. HRM software is then used to identify areas of stable pre- and post-melt fluorescence intensity from the HRM curve (Figure 1A). These signals are then automatically normalized to relative values of 1.0 and 0, respectively (Figure 1B), eliminating differences in background fluorescence and increasing the ability to detect subtle melt profile differences. Both melting temperature shifts and curve shape are used to identify sequence differences. Homozygous allelic/sequence variants are typically characterized by the temperature (x-axis) shift observed in an HRM melt curve (Figure 1, red and blue traces), whereas heterozygotes are commonly characterized by a change in melt curve shape generated from base-pairing mismatches (Figure 1, green trace) generated as a result of destabilized heteroduplex annealing between some of the wild-type and variant strands. Analyzing the data in temperature-shifted view can assist in discriminating these duplex types.

HRM data are often plotted using a difference curve to visually magnify the melt profile differences between different clusters of the same genotype (Figure 1C).

HRM-Compatible Instrumentation

Various designs of HRM-compatible qPCR instruments are available. Access to the following features enables rapid generation of robust HRM data:

- Flexible input sample volume compatible (5–50 µl)
- Standard 96- or 384- well format for increased throughput and streamlined reagent dispensing
- Gradient-enabled thermal block for fast reaction optimization
- No requirement for proprietary dyes, consumables, or reagents
- Fast and uniform thermal control with proven ability and sensitivity to resolve class I through IV SNPs

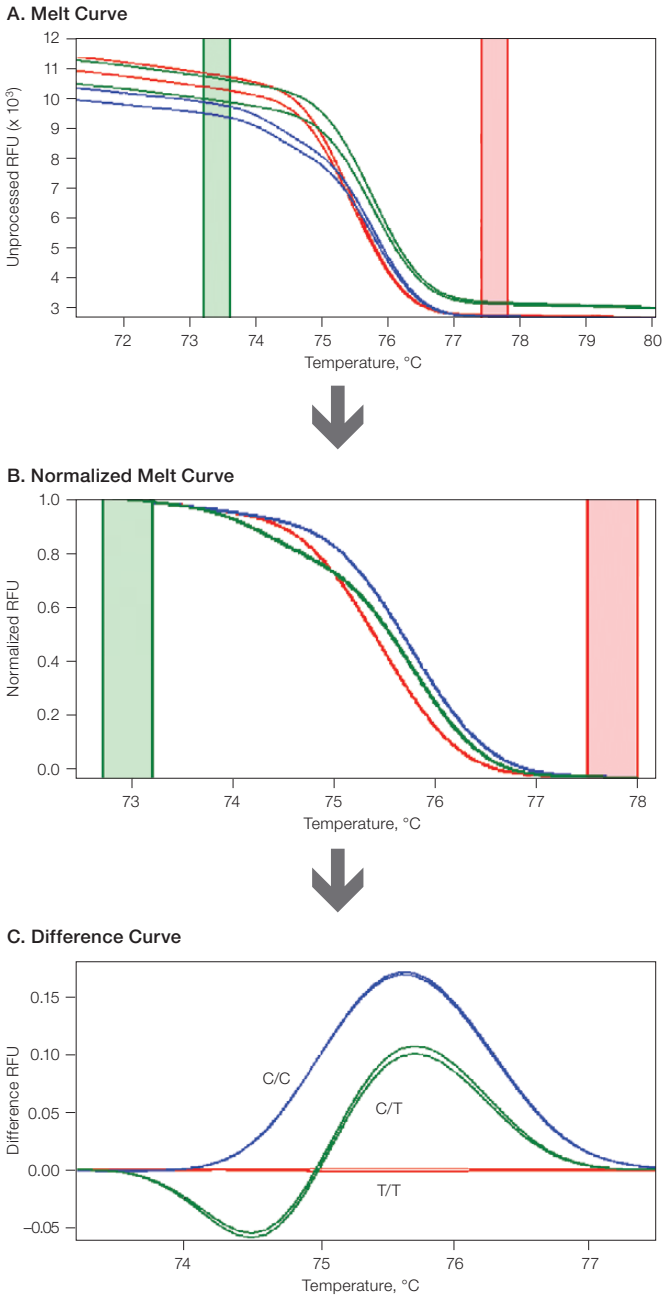

Fig. 1. HRM melt curve analysis. Data were generated using the CFX96™ real-time PCR detection system and analyzed using Precision Melt Analysis™ software. Pre-melt (initial) and post-melt (final) fluorescence signals of all samples (**A**) were normalized to relative values of 1.0 and 0 (**B**). Curve differences were magnified by subtracting each curve from the most abundant type or from a user-defined reference (**C**). RFU, relative fluorescence units.

HRM-Compatible Software

While all qPCR instruments are packaged with analysis software, not all software packages are suitable for HRM analysis. HRM-compatible software should allow the user to:

- Compare and combine data from multiple experiments by combining run results into a single melt study
- Display a plate view for easy identification of sample genotypes
- Share analysis settings among experiments
- Analyze multiple experiments from a single plate
- View all charts in a single window for simplified data analysis and interpretation

Experimental Design Considerations for Successful High Resolution Melt Analysis

Sample Selection and Quality Control

qPCR with HRM analysis is a robust technology but, as with most assays, good sample preparation will result in solid, quantifiable data. To ensure quality data, the following points should be considered when preparing DNA for HRM.

1. Target sample purity with $A_{260/280}$ and $A_{260/230}$ ratios in the range of 1.8–2.2 and 1.6–2.4, respectively, is recommended.
2. DNA integrity should be examined by gel electrophoresis to ensure the presence of undegraded, high molecular weight DNA fragments.
3. Although a DNA sample may display good integrity and apparent purity, appropriate reference gene(s) amplification is beneficial as an internal positive control and to normalize for concentration variation between samples.
4. The EDTA concentration should be minimized in the DNA solution because EDTA can interfere with the activity of some of the enzymes used in the downstream reactions, particularly when at high concentrations (>0.5 mM).

HRM-Compatible Saturating Dyes

HRM analysis relies upon the ability to analyze subtle changes in melt kinetics that typically require a dsDNA intercalating, saturating dye (Figure 2). These dyes are much less inhibitory to PCR than SYBR® Green I and, consequently, can be used at higher (saturating) concentrations to enable consistent and superior discrimination of PCR-amplified DNA (Monis et al. 2005, Mao et al. 2007). Several dye choices are available, the most prevalent being EvaGreen, LCGreen, SYBR® GreenER™, and SYTO 9.

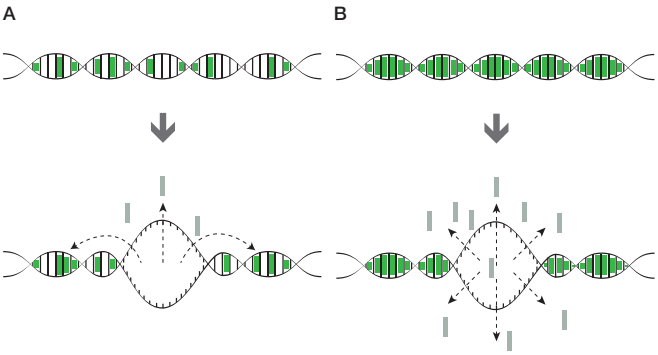

Fig. 2. Saturating and non-saturating dyes. A, when using a non-saturating dye, such as SYBR® Green I, dye molecules can relocate to unmelted regions of dsDNA during melting of DNA, which results in a small or nonuniform temperature shift in the melt curve; **B**, when using a saturating dye, the dye molecules from the melted region cannot relocate to the unmelted portion of the fully saturated amplicon, resulting in melt profiles that more accurately reflect DNA sequence.

Primer Design and Amplicon Length

Analyzing short DNA amplicons can assist with genotype discrimination in HRM experiments. Wherever possible, analyzing amplicons smaller than 100 bp is preferable, especially when sites with a known polymorphism are investigated. It is possible to detect sequence variations with longer amplicons; however, a single base variation influences the melting behavior of a 50 bp amplicon more than a 100 bp amplicon (Figure 3). Reducing amplicon size increases the difference in signal at a given temperature (maximal at the melting temperature [T_m]) between two sequences that differ at only one nucleotide position. This in turn will increase the statistical confidence in calling particular SNPs between samples to give data of higher reliability for publication.

A number of programs are available to help design primer pairs and pick target sequences. Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHomeAd), a freeware option for designing oligonucleotides, is a program developed by the National Center for Biotechnology Information that uses the algorithm Primer3. Primer sequences are compared to the user-selected databases to ensure they are unique and specific for the gene of interest. Beacon Designer (PREMIER Biosoft International), a commercially available program, is also recommended. Beacon Designer designs HRM-specific primers that flank an SNP of interest so that the shortest possible amplicons with detectable melting temperature variation are generated. It is good practice to design and validate three sets of primers to increase the chances of identifying a highly robust pair.

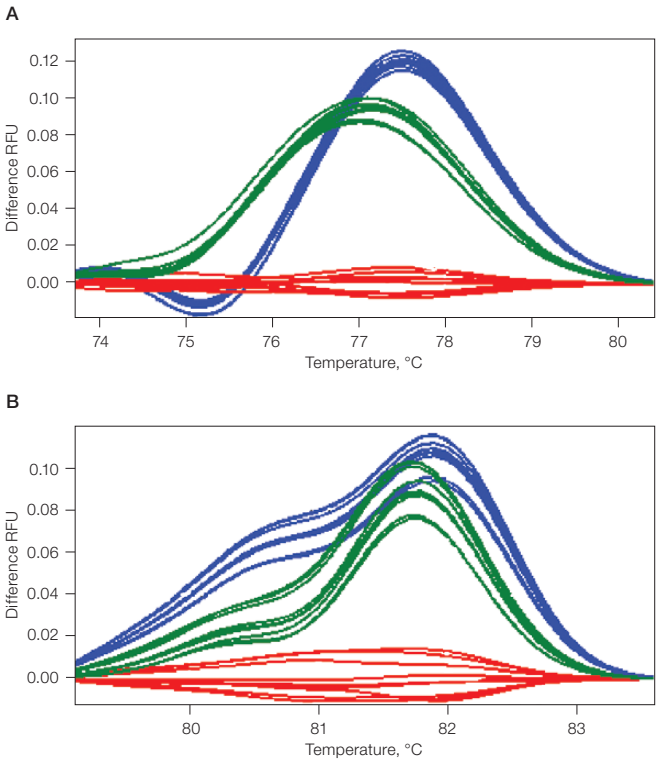

Fig. 3. Effect of amplicon size on melt curves. Difference plots for melt curves of 50 bp (**A**) and 100 bp (**B**) amplicons of wild type (A/A, —), heterozygote (A/T, —), and homozygote SNP (T/T, —) are compared. Clustering and the confidence percentage are increased by using smaller amplicons. Each trace represents a replicate. RFU, relative fluorescence units.

Inconsistent data may be the result of secondary structures in single-stranded or partially denatured DNA. Amplicon sequences should be tested with MFOLD (http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi) to ensure they do not form secondary structures during PCR that can increase the complexity of melting profile interpretation. The DINAMelt server (http://dinamelt.bioinfo.rpi.edu/download.php) is also a useful tool to assess target melt domain complexity.

PCR Reaction Optimization

The success of HRM analysis depends on the quality of the individually amplified PCR products as much as the post-PCR melting protocol and the specific sequence under investigation. Each experimental parameter must be controlled and highly reproducible from sample to sample to ensure successful and reproducible results. Analyzing real-time PCR amplification data prior to HRM analysis can be extremely useful when troubleshooting HRM experiments. Consider the recommended guidelines below for the development of successful HRM experiments.

1. **Amplicon quality.** HRM software may not be able to discriminate nonspecific reaction products if their melt profiles are similar to the target sequence of interest. Ensure only target amplicons of interest are being amplified by generating post-amplification low resolution melt profiles (0.5°C steps) and, ideally, assessing PCR products from each primer pair using agarose gel electrophoresis.

2. **Use sufficient amplification template.** Samples should have a threshold or quantification cycle (C_q) less than 30 cycles. Products that amplify late due to limited starting template amount, template degradation, or PCR inhibition produce variable HRM results.

3. **Normalize input template concentration.** For best results, the amount of template added to the reaction should be consistent. Normalize the starting concentrations so that all amplification plots are within three C_qs of each other, that is, within less than a 10-fold range of one another.

4. **Use an optimized annealing temperature and reaction efficiency.** Annealing temperature has a great impact on reaction specificity and efficiency. Efficient and robust reactions generate reproducible data. Reactions should be optimized using a temperature gradient to determine the appropriate annealing temperature. Test primers and samples for reaction efficiency, which should be between 90 and 110%.

5. **Check for aberrant amplification plots.** Carefully examine amplification data for abnormal amplification curve shapes. A curve with a jagged log-linear phase or one that reaches a low signal plateau compared to other reactions can indicate poor amplification or a fluorescence signal that is too low for analysis. Unsuccessful amplification can be caused by reaction inhibitors, too little dye, or incorrect reaction setup. HRM from such samples can cause low resolution and poor or inconsistent classification.

6. **Normalize input amplicon concentrations.** DNA fragment concentration affects its melting temperature. Ensure every reaction has amplified to the plateau phase for reliable and reproducible melt comparisons.

7. **Ensure sample-to-sample uniformity.** Within an experiment, samples must be of equal volume and contain the same concentration of binding dye. DNA melting behavior is affected by salts in the reaction mix, so the concentration of buffer, Mg^{2+}, and other salts should be as uniform as possible in all samples.

8. **Allow sufficient data collection for normalization of pre- and post-melt regions.** For easier data interpretation and results with tighter replicates, sufficient baseline data points must be collected. This can be easily accomplished by capturing HRM data points over at least a 10°C (or greater) window, centered over the observed melting temperature of the amplified product. A good HRM analysis software, such as Precision Melt Analysis software from Bio-Rad, will help automate most of these processes to ensure consistent data analysis between samples.

Conclusions

HRM is a low-cost, readily accessible technique that can be used to rapidly analyze multiple genetic variants. Careful sample preparation and planning of experimental and assay design are crucial for robust and reproducible results. Following the guidelines above will assist in the development of such assays.

References

Alves DM et al. (2008). Development and use of single nucleotide polymorphism markers for candidate resistance genes in wild peanuts (Arachis spp). Genet Mol Res 7, 631-642.

Dracatos PM et al. (2008). Discovery and genetic mapping of single nucleotide polymorphisms in candidate genes for pathogen defence response in perennial ryegrass (Lolium perenne L.). Theor Appl Genet 117, 203-219.

Frazer KA et al. (2007). A second generation human haplotype map of over 3.1 million SNPs. Nature 449, 851-861.

Garritano S et al. (2009). Determining the effectiveness of high resolution melting analysis for SNP genotyping and mutation scanning at the TP53 locus. BMC Genet 10, 5.

International HapMap Consortium (2005). A haplotype map of the human genome. Nature 437, 1299-1320.

Lander ES et al. (2001). Initial sequencing and analysis of the human genome. Nature 409, 860-921.

Lascorz J et al. (2010). Genome-wide association study for colorectal cancer identifies risk polymorphisms in German familial cases and implicates MAPK signalling pathways in disease susceptibility. Carcinogenesis 31, 1612-1619.

Malentacchi F et al. (2009). Quantitative evaluation of DNA methylation by optimization of a differential-high resolution melt analysis protocol. Nucleic Acids Res 37, e86.

Mao F et al. (2007). Characterization of EvaGreen and the implication of its physicochemical properties for qPCR applications. BMC Biotechnol 7, 76.

Monis PT et al. (2005). Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. Anal Biochem 340, 24-34.

Venter JC et al. (2001). The sequence of the human genome. Science 291, 1304-1351.

Zhao F et al. (2010). Sequencing and genetic variation of multidrug resistance plasmids in Klebsiella pneumoniae. PLoS One 5, e10141.

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