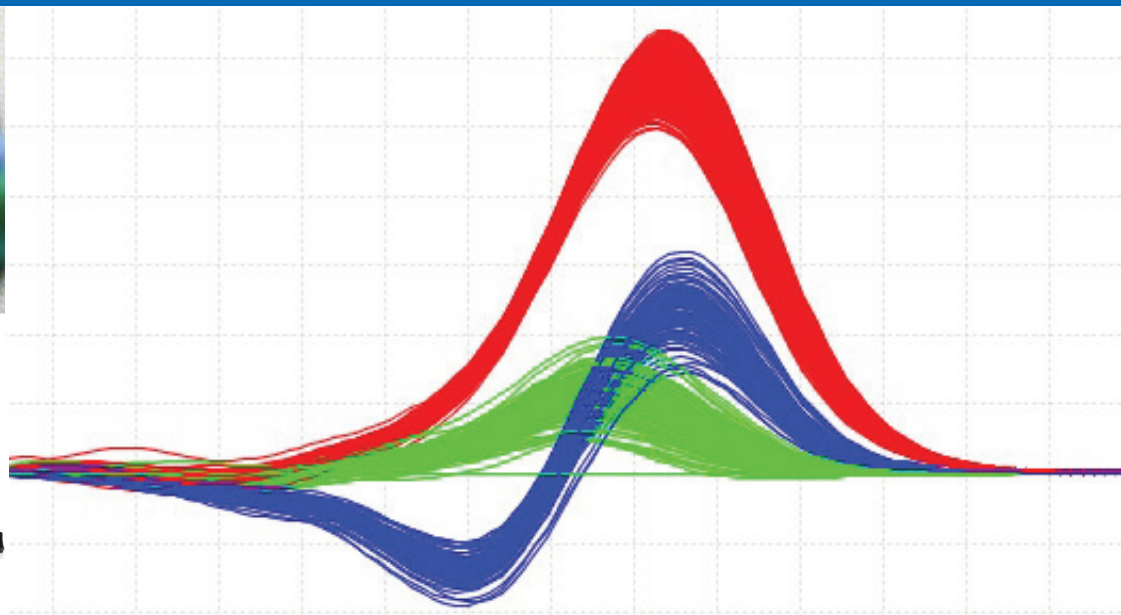


A Guide to High Resolution Melting (HRM) Analysis



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Table of Contents

1. Introduction	3
1.1 About HRM Analysis	4
1.2 Fundamentals of Fluorescent DNA Melting Analysis.....	5
1.3 HRM Analysis	6
1.4 The HRM Analysis Workflow	7
2. HRM Assay and Reagent Optimization	8
2.1 DNA Quality	8
2.2 Amplicon Length	10
2.3 Primer Selection	12
2.4 Dye Selection	13
2.5 HRM PCR Reagents	13
2.6 Instruments and Software	16
3. Troubleshooting Your HRM Experiment	18
4. Summary	19
5. Applications Appendix	20
6. References	20

Sidebars	
Informational	
Amplicon Length and SNP Detection	10
Human Single Nucleotide Polymorphisms (SNPs) Occurrence and T_m	11
SYBR® Green Dye Use in HRM Analysis	13
Definition	
Autocalling, Variant Calls	8
Tip	
HRM Analysis of Challenging DNA Samples	9
High-Quality DNA Key to Successful HRM Analysis	9
DNA Quality Considerations for Successful HRM Analysis	9
Primer Design for Successful HRM Analysis.....	12
Ideal Amplification Properties for Successful HRM Analysis	14
Optimizing PCR Reagents for Successful HRM Analysis.....	15
Other Suggestions for Successful HRM Analysis	15
Other Resources	
General HRM	3
Other Troubleshooting Resources.....	18
Product	
Primer Design Software.....	12
Shopping List for HRM Analysis—Suggested Reagents.....	15
Real-Time PCR Systems for HRM Analysis	16

1. Introduction

1. Introduction

High Resolution Melting (HRM) analysis is a new, post-PCR analysis method used for identifying genetic variation in nucleic acid sequences. Simple and fast, this method is based on PCR melting (dissociation) curve techniques and is enabled by the recent availability of improved double-stranded DNA (dsDNA)-binding dyes along with next-generation real-time PCR instrumentation and analysis software. HRM analysis can discriminate DNA sequences based on their composition, length, GC content, or strand complementarity [1].

HRM analysis differs from standard melt curve analysis in three ways:

1. **Chemistry**—HRM analysis uses brighter dyes at higher concentrations
2. **Instruments**—HRM analysis requires instruments that collect fluorescence data at finer temperature resolution
3. **Software**—HRM analysis requires more sophisticated software which uses new fluorescent scaling algorithms and plots

HRM analysis starts with PCR amplification of the region of interest in the presence of a dsDNA binding dye. This binding dye has a high fluorescence when bound to dsDNA and low fluorescence in the unbound state. Amplification is followed by a high resolution melting step using instrumentation capable of capturing a large number of fluorescent data points per change in temperature, with high precision. When the dsDNA dissociates (or melts) into single strands, the dye is released, causing a change in fluorescence. The result is a melt curve profile characteristic of the amplicon.

HRM analysis requires no manual post-PCR processing, is performed in a closed-tube system, and has a low reaction cost relative to other methods used to study genetic variation.

HRM is a relatively new method and this guide provides a resource for scientists learning and perfecting the technique. Along with an introduction to HRM analysis, information is provided on reaction optimization and technical problem solving.

Other Resources—General HRM

- Applied Biosystems HRM Analysis Site—www.appliedbiosystems.com/hrm
- *Applied Biosystems High Resolution Melting Software Getting Started Guide* (PN 4393102; view at www.appliedbiosystems.com) provides detailed, step-by-step procedures for HRM calibration and analysis using Applied Biosystems instruments and HRM software
- *Applied Biosystems High Resolution Melting Software Help* (PN 4393101; view at www.appliedbiosystems.com) describes HRM analysis software and procedures for use
- Oligo Calculators to quickly calculate T_m , %GC, molecular weight, and other characteristics of oligonucleotides
 - Applied Biosystems oligonucleotide calculator www.appliedbiosystems.com/support/techtools/tm_calculator.cfm
 - OligoCalc—www.basic.northwestern.edu/biotools/oligocalc.html
- PCR design software
 - Primer Express® software, included with Applied Biosystems instruments—www.appliedbiosystems.com/primerexpress
 - Methyl Primer Express® software—www.appliedbiosystems.com/methylprimerexpress
 - Primer3—<http://frodo.wi.mit.edu/>
 - Primer3Plus—www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi
- Gene Quantification Information—www.gene-quantification.de/hrm.html

1.1 About HRM Analysis

In HRM analysis, the region of interest is amplified by PCR in the presence of a fluorescent dsDNA binding dye. Following PCR, the product is gradually melted, and the emitted fluorescence is measured on a specialized instrument to generate a characteristic curve. The resulting melt profile reflects the mix of amplicons present. Aspects such as GC content, length, sequence, and heterozygosity will add to the melt curve characteristics for each amplicon. The resulting profiles can provide valuable information for mutation screening, genotyping, methylation, and other investigative applications.

HRM analysis uses two profile observations:

1. Melt curves that are similar in shape but that are distinguishable from each other by difference in melting temperature (T_m) of the amplicon. Typically such profiles are generated by homozygous variant samples that are being compared to a wild type sample. In such situations, the T_m difference between samples is due to sequence variation from the wild type.
2. Melt curves displaying a distinct curve shape from homozygote melt curves. These profiles are usually due to the presence of base pairing mismatches (heteroduplexes) present in the PCR product mix.

Figure 1 provides an example of HRM analysis data to illustrate the different types of resulting profiles.

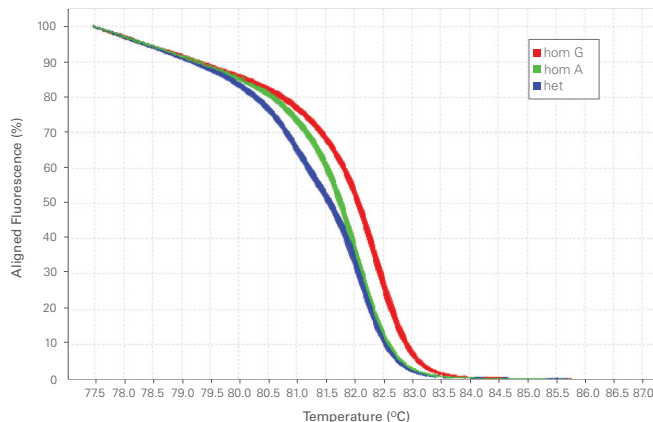


Figure 1. High Resolution Melt Curve With a Single Point Mutation. This is an aligned melt curve. The plot demonstrates the sharp decrease in fluorescence when the double-stranded DNA melts into its single-stranded form. Red = homozygous with guanine, Green = homozygous with adenine, Blue = heterozygous.

1.2 Fundamentals of Fluorescent DNA Melting Analysis

Standard DNA melt curve analysis is a post-PCR analysis method based on a biophysical measurement of the amplified DNA. Historically, it has been used for various applications, most commonly to detect primer-dimers or other nonspecific by-products of PCR.

A melt curve is generated by slowly denaturing (melting) the DNA sample through a range of temperatures in the presence of a dsDNA binding dye. During melting, only dye bound to dsDNA fluoresces, and that fluorescence is continuously detected by an optical system. Fluorescence is high when the dye is in its bound state, but when in solution, the dye changes conformation and fluorescence decreases 1,000-fold.

The extent of melting is measured by the change in fluorescence of the DNA sample. At low temperatures, the DNA will be double-stranded and the dye will strongly fluoresce. As the temperature increases, fluorescence will decrease 1,000-fold as the dye is released when the two strands denature. This decrease in fluorescence starts slowly; but when the double-stranded DNA melts into its single-stranded, fully denaturated form, a sharp decrease in fluorescence is detected (Figure 2A). The rate of fluorescence decrease is generally greatest near the

melting temperature (T_m) of the PCR product. The T_m is defined as the point in the melt curve where 50% of the DNA is double-stranded, and 50% is single-stranded (melted). It equals the temperature at which the aligned fluorescence is 50% and is a function of PCR product characteristics, including GC-content (T_m is higher in GC-rich PCR products), length, and sequence content.

Melt curve raw data is generally plotted as fluorescence versus temperature. In general, melt curve analysis software defines the T_m of a PCR product as the inflection point of the melt curve. To visualize the T_m more clearly, the negative first derivatives are often plotted, making the T_m s of the products appear as peaks, as seen in Figure 2B. Nonspecific products may also appear in the derivative curve. These peaks are typically of lower intensity and represent products that are shorter in length, thus appearing at a lower temperature than the primary product. Because primer-dimers and other nonspecific PCR products can be seen in this data view, it can be a useful measure of PCR product purity.

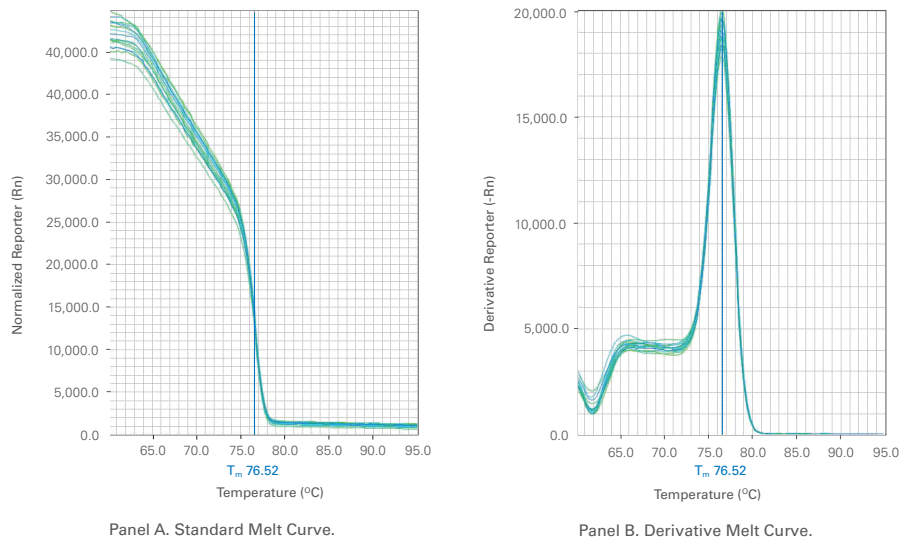


Figure 2. Real-Time PCR Data Showing a DNA Melt Profile. (A) A standard melt curve plot, showing the high initial fluorescence when all products are double-stranded and the maximum amount of dye is bound. As the temperature increases, the PCR products denature, dye is released, and the fluorescent signal drops. The vertical line is the point where 50% of the PCR products in the sample are denatured. (B) A derivative melt curve plot, which shows the inflection point on the slope as a more easily visualized melt peak.

1.3 HRM Analysis

In HRM analysis, the same fundamental principles of DNA melt curve analysis are applied along with further refinements. Complex alignment algorithms and new plot views are introduced so that small differences in melt curve data can be seen.

Figure 3 illustrates the typical process of data alignment using the Applied Biosystems® HRM Software v2.0. Pre- and post-melt regions are defined by pairs of vertical bars placed before and after the active melt region. The area of data between the pair of bars to the left of the active melt region is used by the software to designate 100% fluorescence—where every amplicon is double-stranded. This level is shown at the far left of the aligned melt curve in Figure 3.

The change in fluorescence for each sample, seen to the right of the 100% fluorescence point, is scored as the true fluorescence change and

is used to plot the aligned melt curve (Figure 3). The post-melt region is delineated by the pair of parallel lines to the right of the active melt region, and is used by the software to determine the 0% fluorescence point—where every amplicon is single-stranded.

The aligned plot provides a scaled view of the data, enabling easy discrimination of sequence variants that display true differences in their melt curve behavior. This is in contrast to the raw data that is affected by the variable intensity of fluorescence between each sample.

Often, differences between melt curves are small. Small differences are best visualized using a difference plot (Figure 4). In this plot, sample curves are subtracted from a single reference (typically a wild-type control), run in the same experiment.

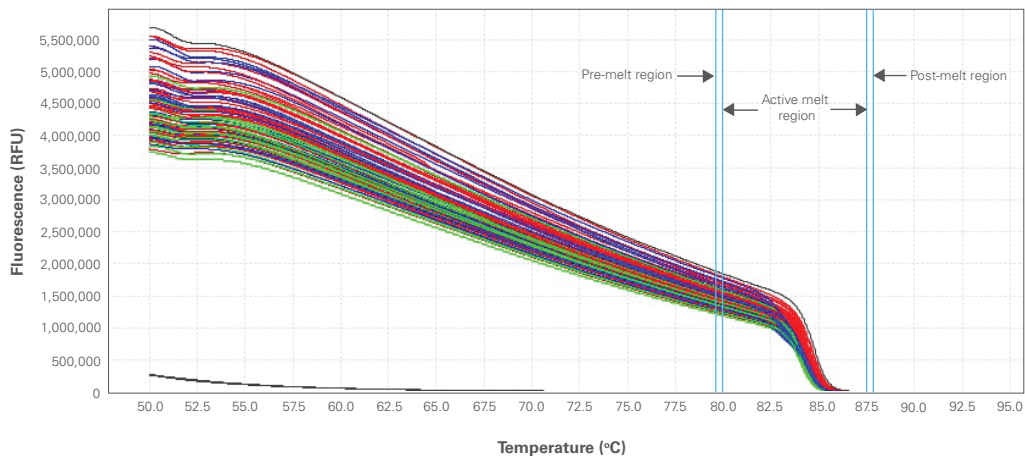


Figure 3. Raw Data Melt Curve. Data collected during a high resolution melt curve experiment exhibits a range of initial fluorescence readings, making it difficult to discriminate differences in the change of fluorescence for individual samples. The active melt region is designated by pre- and post-melt regions (double bars) that are used to align the data, producing a clearer view of the melt curve results. (Curve colors represent different samples.)

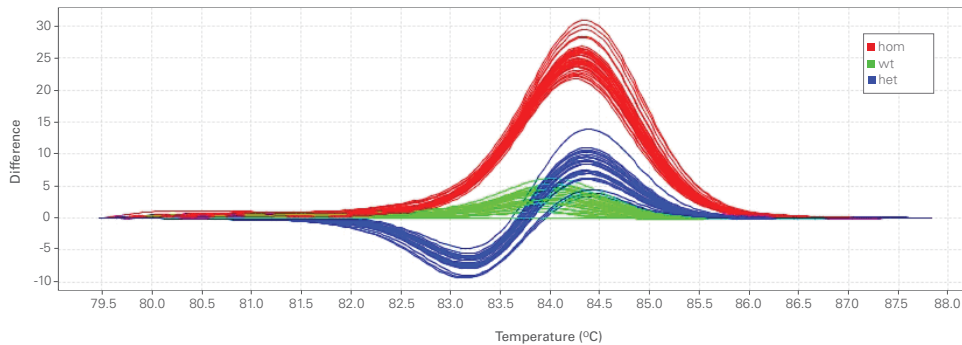


Figure 4. HRM Analysis Data Difference Plot. Sample curves are subtracted from a reference curve (usually a wild-type control). This view accentuates differences between similar melt curves. Green = wild type, Red = homozygous, Blue = heterozygous.

1.4 The HRM Analysis Workflow

The actual HRM analysis workflow is simple (Figure 5); most of the work lies in the optimization of the PCR primers, reagents, and reaction conditions (Step 2).

Step 1. Instrument Calibration (for Applied Biosystems® 7500 Fast or 7900HT Fast Real-Time PCR Systems).

The instrument used for the PCR amplification and HRM analysis should be calibrated once every six months. This step includes running a background, custom dye, and HRM calibration plate. For more information on the calibration procedure, refer to *Applied Biosystems High Resolution Melting Software Getting Started Guide* (PN 4393102; view or order from www.appliedbiosystems.com).

Step 2. PCR Design and Optimization.

This step requires the most attention. It includes careful design of primer sets, and the selection and testing of PCR reagents, HRM dyes, and reaction controls (see *PCR Assay Development & Optimization*, page 8, for more information).

Step 3. PCR.

Use of a real-time PCR system for amplification is recommended, particularly when optimizing or troubleshooting an assay, as PCR amplification can be monitored in real time, providing information on amplicon quality. The data can also be checked for melt analysis suitability and saved in the appropriate file format (see *PCR Assay Development & Optimization*, page 8, for more information). When a real-time PCR system is used, Steps 3, 4, and 5 may be completed on the same instrument. Alternatively, PCR may be performed on a standard PCR thermal cycler followed by transfer to a real-time PCR system for HRM.

Step 4. Sample Melt.

On a real-time PCR system, samples are melted and the data collected. When a standard thermal cycler is used for the PCR, the samples must be transferred to a real-time PCR instrument designed to collect HRM melt curve data.

Step 5. Data Analysis.

The PCR data file is imported into the HRM analysis software and the data is analyzed.

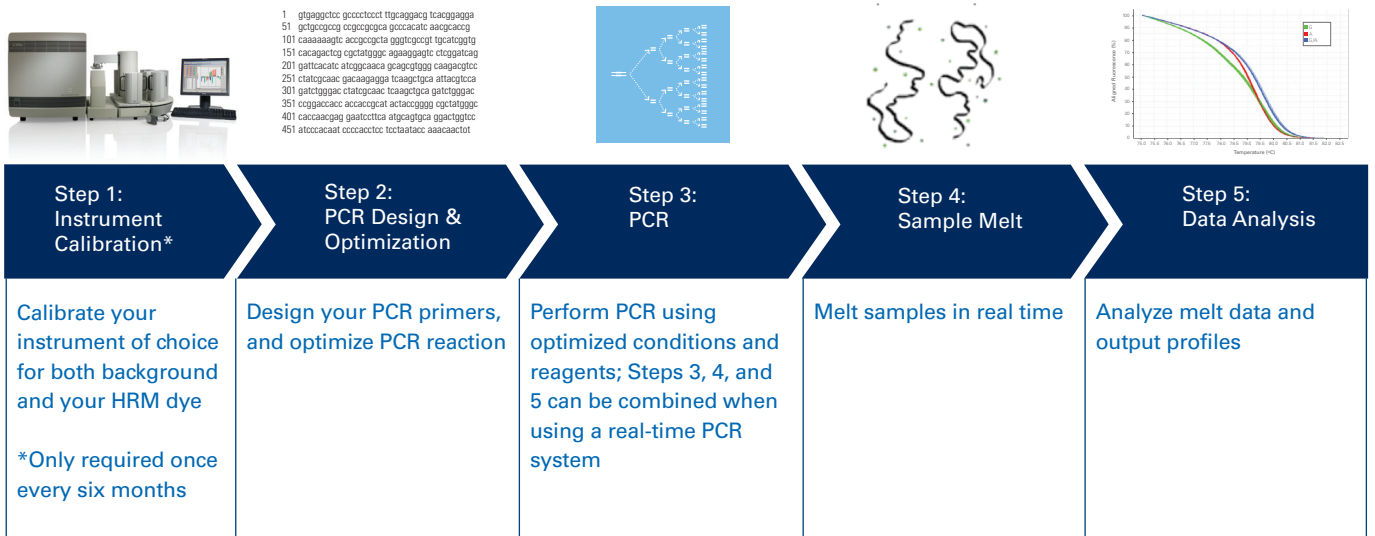


Figure 5. The HRM Analysis Workflow.