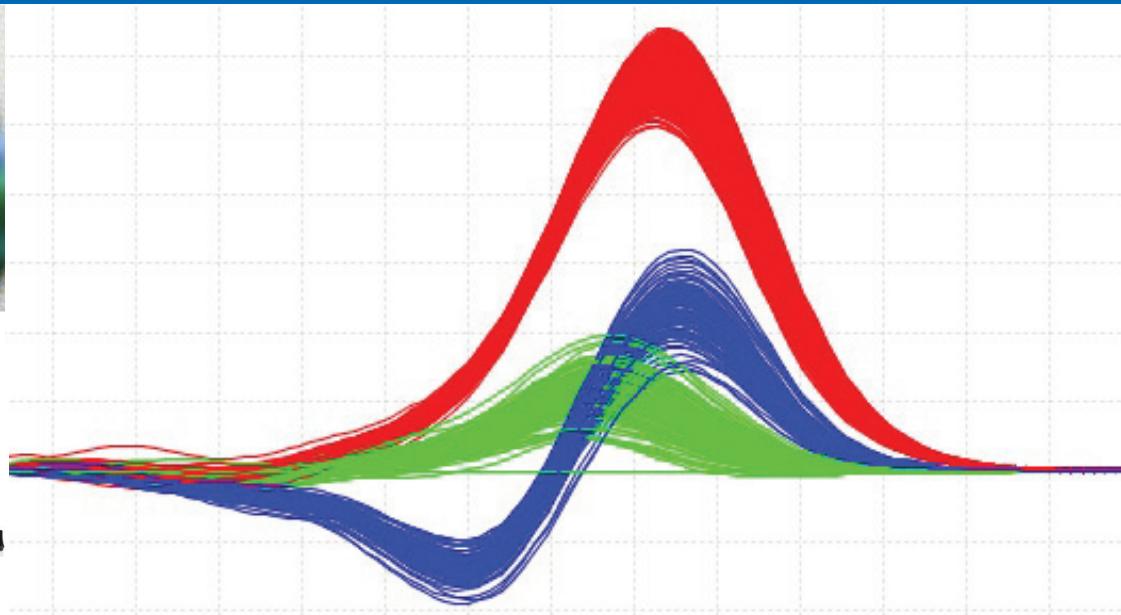


A Guide to High Resolution Melting (HRM) Analysis



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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.

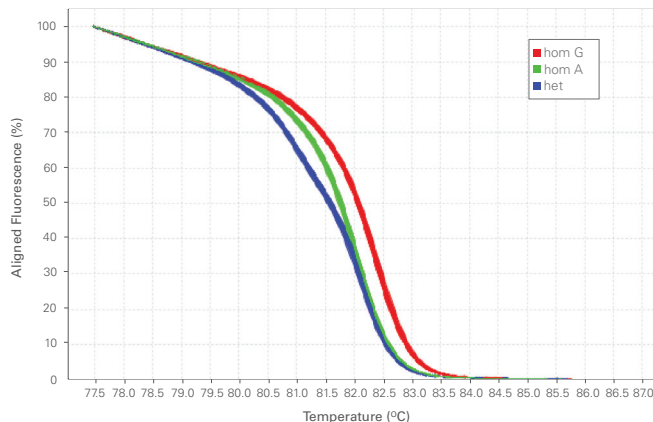


Figure 1. High Resolution Melt Curve With a Single Point Mutation. This is an aligned melt curve. The plot demonstrates the sharp decrease in fluorescence when the double-stranded DNA melts into its single-stranded form. Red = homozygous with guanine, Green = homozygous with adenine, Blue = heterozygous.

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.

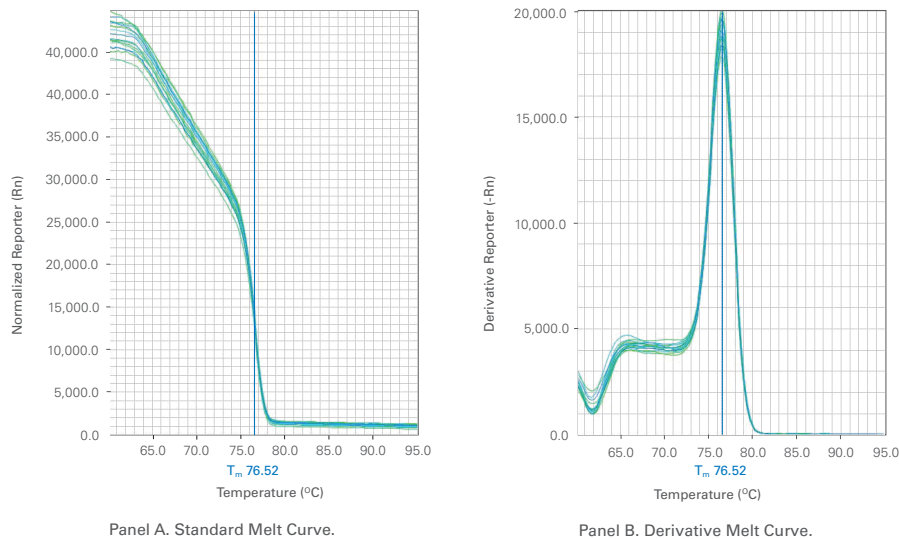


Figure 2. Real-Time PCR Data Showing a DNA Melt Profile. (A) A standard melt curve plot, showing the high initial fluorescence when all products are double-stranded and the maximum amount of dye is bound. As the temperature increases, the PCR products denature, dye is released, and the fluorescent signal 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.

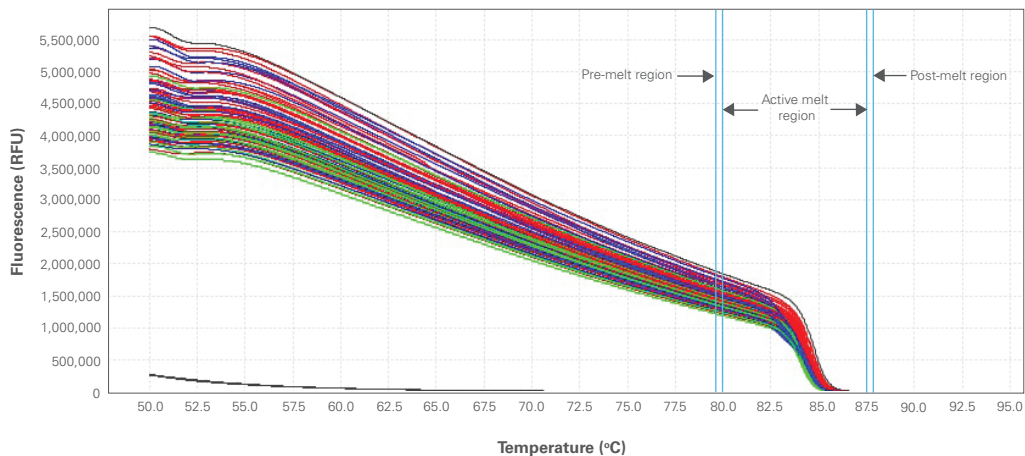


Figure 3. Raw Data Melt Curve. Data collected during a high resolution melt curve experiment exhibits a range of initial fluorescence readings, making it difficult to discriminate differences in the change of fluorescence for individual samples. The active melt region is designated by pre- and post-melt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

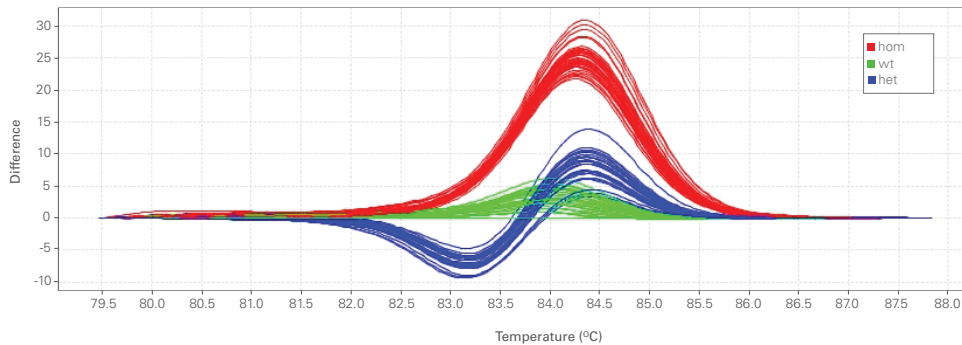


Figure 4. HRM Analysis Data Difference Plot. Sample curves are subtracted from a reference curve (usually a wild-type control). This view accentuates differences between similar melt curves. Green = wild type, Red = homozygous, Blue = heterozygous.

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.

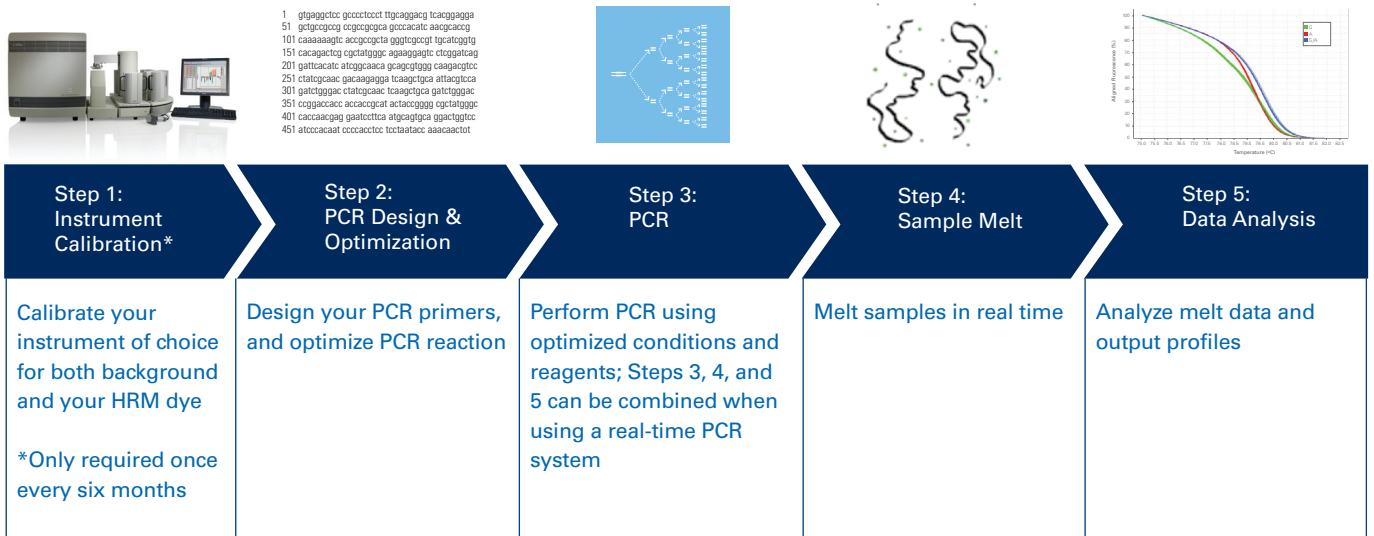


Figure 5. The HRM Analysis Workflow.

2. HRM Assay and Reagent Optimization

2. HRM Assay and Reagent Optimization

For successful analysis, care should be taken in assay development to ensure that the experiment is optimized for HRM. Small differences in melt curves can arise from sources other than sequence, such as genomic DNA (gDNA) quality, carryover of extraction impurities, amplicon length, primer design, dye selection, and PCR reagent choice. The following sections address these factors.

Definition

Autocalling—An automated grouping of sequences deemed by the analysis software to have similar HRM curve shapes and T_m .

Variation Calls—Groups of sequences determined by the software analysis parameters to have similar mismatches, insertions, or deletions distinct from any user-defined controls. These groups are designated variant 1, variant 2, etc.

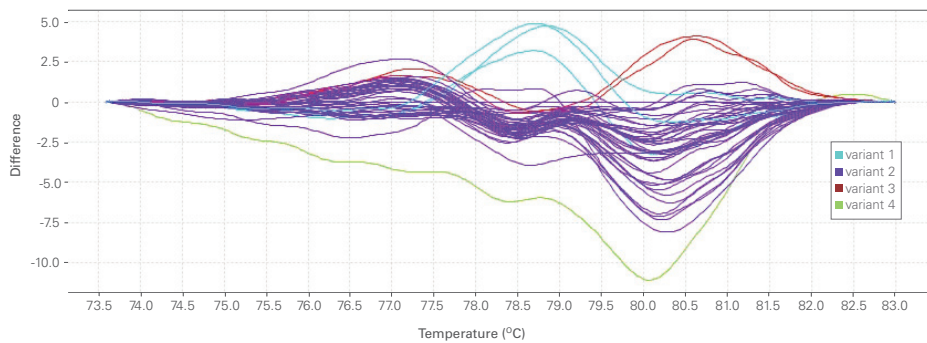
2.1 DNA Quality

One of the biggest factors associated with DNA quality that can impact HRM results is salt carryover, as it will subtly change the thermodynamics of the DNA melting transition. If not controlled for, salt carryover and other experimental artifacts can complicate the interpretation of DNA melt curves, leading to lower reproducibility and higher error rates in HRM variant calls.

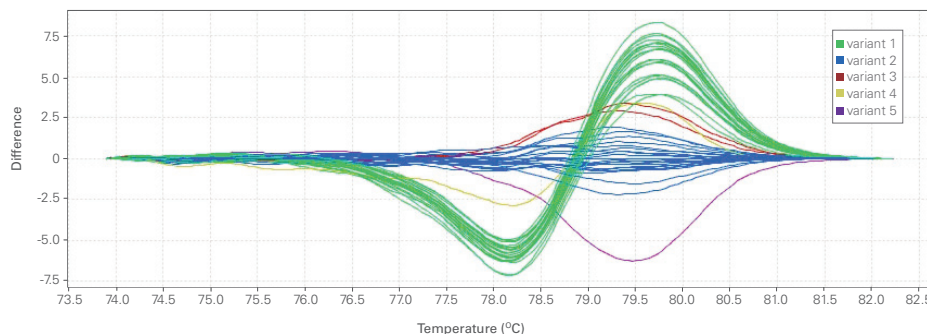
Some commercial DNA isolation kits use high salt (1M) for nucleic acid elution from columns or filters. These samples will need to be precipitated and resuspended in a low salt buffer such as TE (10 mM Tris, 1 mM EDTA) prior to PCR. pH extremes resulting from NaOH usage in isolation procedures can also carry over and impact HRM analysis. Precipitation and resuspension in TE can remedy this. Dilution of the sample in both situations can also help.

Panel 6A shows the difference plot obtained from amplification of poor-quality DNA samples. The extraction method used for these samples resulted in carryover of PCR contaminants and salts which had dramatic effects on the melt curves. The multiple peaks and large spread of the amplicon populations creates a complex pattern that is difficult to interpret.

Panel 6B shows a difference plot of the same DNA samples from 6A, having undergone dilution prior to PCR amplification. The decreased salt and other contaminants in the PCRs resulted in melt profiles that were smoother, more tightly grouped, and easier to separate into clear clusters.



Panel 6A. Difference Plot of Salt-Contaminated DNA Samples.



Panel 6B. Difference Plot of Diluted, Salt-Contaminated DNA Samples.

Figure 6. Difference Plots of Contaminated DNA Samples. DNA sample variants were isolated using a commercial isolation kit employing a high salt wash, resulting in salt-contaminated DNA. Samples were split, and half were diluted 1:100 in water. Difference plots of HRM analysis of the (A) undiluted and (B) diluted samples are shown, illustrating how dilution of a contaminated sample can often increase data quality.

2. HRM Assay and Reagent Optimization

Low-quality DNA may produce nonspecific PCR products, ultimately resulting in incorrect scoring of results in HRM analyses, or failed reactions. Samples that amplify late or fail to reach a high signal plateau in the PCR phase can result in inconclusive or low-resolution HRM data [2].

For the best results, all DNA samples in an analysis run should be prepared using the same method. DNA input amount into the PCR should also be similar. Furthermore, HRM analysis should be performed immediately following PCR whenever possible. If immediate analysis is not possible, the PCR products can be stored at -20°C for subsequent remelting, reamplification, or sequencing.

Tip: HRM Analysis of Challenging DNA Samples

More challenging DNA samples can be used successfully in HRM analysis when combined with robust reagents and instrumentation. For example, genomic DNA isolated from formalin-fixed, paraffin-embedded (FFPE) tumors has been used successfully to identify SNP variants (see *Identifying Variants by High Resolution Melting (HRM) in Difficult Samples*, Chapter 5 page 20; also *DNA Genotyping from Human FFPE Samples—Reliable and Reproducible*; publication 137AP04-01, available at www.appliedbiosystems.com).

Tip: High-Quality DNA Key to Successful HRM Analysis

In a recent study, scientists amplified 70 DNA samples of varying quality in an attempt to determine the effect on HRM analysis. The DNA samples were amplified using a multiplexed control gene primer set designed to produce products of 100, 200, 300, 400, and 600 bp with equal intensity. Loss of the higher molecular weight products indicated that the DNA sample was of poor quality. HRM data was correlated with the DNA quality and amplification plot data; poor DNA quality was shown to have a negative impact on the quality of the HRM analysis. Therefore, care should be taken to prepare full-length DNA when at all possible. It is also recommended that the amount of DNA template used in PCR be consistent between samples since large differences in starting template can impact the resulting T_m . (National Genetics Reference Laboratory (Wessex) Technology Assessment. 2006 www.ngrl.org.uk.)

Tip: DNA Quality Considerations for Successful HRM Analysis

- Use the same method to prepare DNA from all samples in an experiment
- Use the same reagents during DNA isolation, including the sample buffer salt concentration, for all samples and controls in the experiment
- Watch for contamination carryover from the DNA purification procedure
- Use similar DNA input amounts for all samples

2.2 Amplicon Length

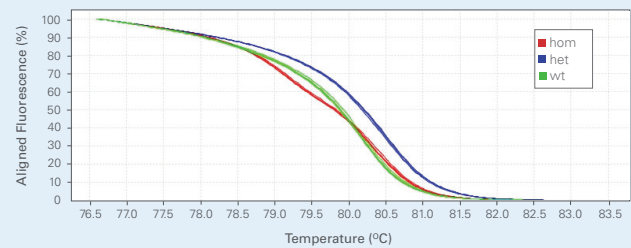
Amplicon lengths of 100–300 bp are generally recommended for HRM analysis of different sequence variants, including single nucleotide polymorphisms (SNP's), inversions, insertions, and deletions. While large sequence variations between amplicons can be detected for amplicons longer than 300 bp, more subtle variations such as A/T class 4 SNPs typically require smaller amplicons for detection.

SNP Genotyping

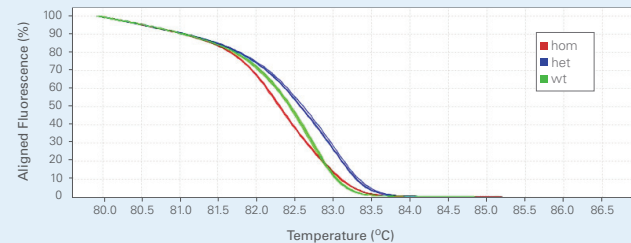
When designing primers for HRM SNP genotyping analysis, the amplicon length should be short to avoid the detection of SNP variants outside of the region of interest. However, smaller amplicons may produce lower fluorescence signals, presumably due to less dye incorporation in the shorter sequence. In order to ensure adequate dye fluorescence signals, the amplicon length should be approximately 80–100 bp including primers (target sequence length of 30 bp in addition to two 25 bp primer sequences).

Informational: Amplicon Length and SNP Detection

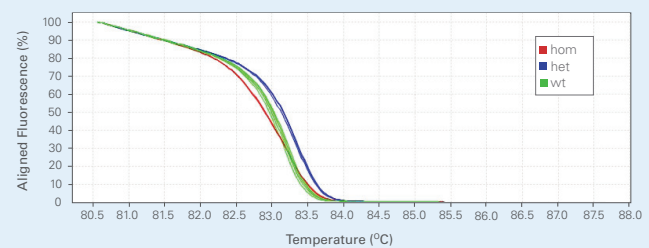
This data shows how amplicon lengths ranging from 120–313 bp can impact HRM analysis for SNP detection (a single point mutation of A/G). Applied Biosystems® HRM Software v2.0 can correctly identify the two homozygous and the heterozygous mutants at all the amplicon lengths tested, however there are distinct differences in the resolutions of the mutants. The clearest discrimination of the mutants is seen when the shortest amplicon (120 bp) is used and the T_m difference between the two homozygotes is near 0.5°C. The distinction between the two homozygous populations begins to diminish when the amplicon is 220 bp and continues to diminish as amplicon size increases. As amplicon size increases, it is possible that additional mutation sites will be detected, which will result in a more complex melting pattern where it becomes more difficult to distinguish between the different sequence variants (not shown).



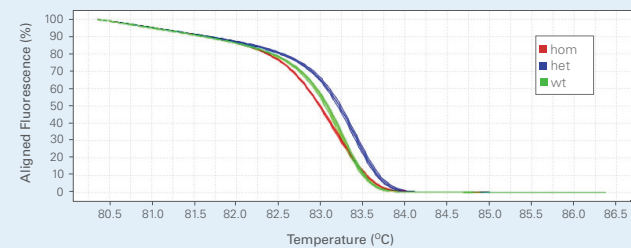
Panel A. 120 bp Amplicon



Panel B. 220 bp Amplicon



Panel C. 233 bp Amplicon



Panel D. 313 bp Amplicon

Figure 7. Effect of Increasing Amplicon Size on HRM Analysis.

2. HRM Assay and Reagent Optimization

Mutation Screening

When using HRM analysis to screen for unknown mutations, the entire gene length needs to be screened, and the amplicon length used can be long, typically 300 bp. The genomic region of interest should be divided into amplicons with lengths <250 bp, which will produce 300 bp amplicon sequences when 25-mer forward and reverse primers are used. Target sequences should overlap by at least 25 bp to ensure complete coverage of the region of interest (Figure 8).

Informational: Human Single Nucleotide Polymorphisms (SNPs) Occurrence and T_m

Table 1 lists the SNPs found in the human genome, their classification, expected T_m between the two homozygous populations of each sequence variant, and their percentage occurrence. Class 1 and 2 SNPs are easier to detect by HRM analysis and occur at a higher percentage in the human genome compared with the more difficult-to-distinguish Class 3 and 4 SNPs.

SNP Class	Base Change	Typical T_m Curve Shift	Occurrence in the Human Genome
1	C/T & G/A	Large (>0.5, avg. of 1.0°C)	64%
2	C/A & G/T	Large (>0.5, avg. of 1.0°C)	20%
3	C/G	Small (0.2–0.5°C)	9%
4	A/T	Very small (<0.2°C)	7%

Table 1. Human Single Nucleotide Polymorphisms (SNPs) Occurrence and T_m .



Figure 8. Effect of Increasing Amplicon Size on HRM Analysis.

2.3 Primer Selection

Because the dyes used in HRM analysis bind generically to any double-stranded DNA products, PCR primers need to be designed for robust performance and must be specific to the region of interest. A common check of specificity is a BLAST search. The Basic Local Alignment Search Tool (BLAST) on the NCBI site (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) quickly checks your primers for regions of similarity to other known sequences.

It is good practice to design three sets of primers. Having multiple primer sets to choose from helps to ensure you will have at least one highly robust pair. Each primer pair should be tested for specificity to the target of interest and produce a minimum of primer-dimers and nonspecific products. PCR followed by melt curve analysis should be performed to look for nonspecific products and primer-dimers. In addition to the standard analysis, melt profiles should also be viewed in low resolution to see these other products. HRM software may not be able to detect all nonspecific reaction products if their melt profiles are similar. As a consequence, it is best practice to initially assess PCR products from each primer pair by agarose gel electrophoresis.

Once the best primer set is chosen, other reaction factors can be further optimized. It is advisable to perform HRM analysis on a real-time PCR instrument for the built-in PCR quality check the instrument can provide (see section 2.6, *Instruments & Software*, page 16, for additional information). If the proper pilot studies have been done for the chosen primer set, the real-time reactions will provide information about DNA template quantity and quality.

Length of the resulting DNA amplicon may also impact the sensitivity and specificity of subsequent HRM analysis. One study found that mutations were more difficult to detect in amplicons greater than 400 bp [2]. Melt profiles generated by shorter amplicons tend to be less complex than those generated by longer amplicons; as a result, shorter amplicons are easier to analyze. The relative change in fluorescence intensity of smaller amplicons is greater than for longer amplicons; however, care should be taken to not make the amplicons too short, as the overall fluorescence will be reduced when there is less double-stranded product to which the dye can bind. For more information, see *Amplicon Length* in section 2.2, found on page 10.

Tip: Primer Design for Successful HRM Analysis

- Use amplicons with a target length of <300 bp to ensure sensitive detection of sequence variants
- Check primer sequences for known variations, annotations, and for species specificity
- Order three primer sets to empirically determine the most robust pair
- Primer T_m should be between 58–60°C
- HPLC purification of primers may help troublesome reactions
- A variety of software tools are available to help design HRM primers, including Primer Express® 3.0 Software, Primer3, and Primer3Plus

Product: Primer Design Software

Primer Express® 3.0 Software

The Applied Biosystems® Primer Express® 3.0 Software is a primer and probe design tool made specifically for use with Applied Biosystems® Real-Time PCR Systems. Primer Express® allows you to independently design oligonucleotides for PCR applications.

The software is simple to use. First, a file of the DNA sequence of interest is imported into the program. Use the default settings for TaqMan® design and select “find primers/probes now”. All acceptable primer and probe sets for your sequence of interest are returned. The software format results in a text document that can be copied into an email for easy primer ordering. Because HRM analysis does not use TaqMan® probes, that information is simply discarded.

To design primers for HRM analysis of genotypes with known mutations, use the TaqMan® MGB Allelic Discrimination Design Document in Primer Express® 3.0 and input the SNP information as if designing a TaqMan® SNP Genotyping Assay.

Figure 9 shows a sample melt profile of a Class 1 SNP (dbSNP rs1801280) for the N-acetyltransferase 2 gene. The primer set was designed using the default settings in Primer Express® 3.0 and analyzed in the HRM Software v2.0.

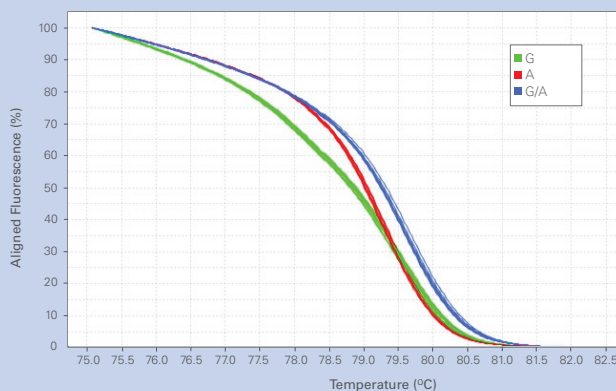


Figure 9. Melt Profile of Class 1 SNP. Primers were designed using Primer Express® Software 3.0.

Methyl Primer Express® Software v1.0—Primer Design for MS-HRM Analysis

HRM analysis can also be used to detect methylation differences between genes; this is known as methylation-specific high resolution melting (MS-HRM) analysis (see Methylation-Sensitive High-Resolution Melting Analysis on 7500 Fast Real-Time PCR System, Chapter 3, page 17). It is known that PCR primers are biased toward nonmethylated fragments; as a result, nonmethylated fragments amplify more efficiently than the methylated fragments, affecting assay sensitivity, particularly at the lower limits of detection. To accurately detect methylation in the 0% to 2% range, include CpG dinucleotides in the PCR primer sequences. CpG sequences bias the amplification towards the methylated fragments of DNA, but can negatively affect the sensitivity of detection. Using the Methyl Primer Express® Software v1.0, primers can be designed with a specified number of CpG dinucleotides for optimized reactions. This free software is available for download at www.appliedbiosystems.com/methylprimerexpress.

2.4 Dye Selection

HRM analysis uses double-stranded DNA (dsDNA)-binding dyes that, at relatively high concentrations, do not inhibit PCR [3]. The amount of fluorescent dye bound to double-stranded DNA can vary with amplicon length, composition, and PCR conditions. Too much dye can stabilize the double-stranded form, artificially shifting the T_m or inhibiting PCR; too little dye can result in low signal and inaccurate results. Ideally, the optimal dye concentration should be determined by empirical testing with a titration experiment. Dye concentrations slightly higher and lower than the recommended starting concentration should be tested to determine the concentration that gives maximum fluorescence without inhibiting the PCR.

These dyes are photosensitive and should be protected from light when not in use. Some dyes are not stable in their diluted form, so fresh dilutions must be prepared for each experiment. The manufacturer's instructions should be closely followed for storage and dilution.

The dyes used for HRM analysis are double-stranded DNA-binding dyes that are brighter than those previously used, and do not inhibit PCR at high dye concentration. Dyes commonly used for HRM analysis include:

- SYTO® 9 Green Fluorescent Nucleic Acid Stain
- EvaGreen® Dye
- SYBR® Green I
- SYBR® GreenER™ Dyes (see sidebar, *SYBR® Green Dye Use in HRM Analysis*, for restrictions)

Whichever dye is chosen, the reaction should be optimized for that specific dye; as each dye interacts uniquely with all other reaction components, impacting the ultimate sensitivity of the analysis.

2.5 HRM PCR Reagents

Reagents for HRM analysis and reaction conditions should be optimized to result in robust and specific PCR. Primer-dimers and other nonspecific products can significantly decrease performance of the HRM analysis; so in addition to optimizing reactions, one must ensure that variation is not introduced by improper assay design and optimization decisions.

Use PCR reagents that have been validated or optimized for HRM analysis, when available. Hot start enzymes, such as that included in the AmpliTaq Gold® 360 Master Mix, are also recommended as they increase primer specificity.

Table 2 shows a basic PCR setup for HRM analysis. Starting with these recommendations, the reaction can be further optimized by testing for optimal primer sets, $MgCl_2$ concentration, template concentration, and other components.

Informational: SYBR® Green Dye Use in HRM Analysis

Standard PCR-based melting (dissociation) curve analysis was introduced about a decade ago and originally used SYBR® Green I dye as the dsDNA binding dye [1]. Until recently, SYBR® Green I dye has been discouraged for HRM analysis because it has been shown to inhibit PCR at high concentrations [4] and has been hypothesized to redistribute from melted regions back into the dsDNA amplicon [2,5]. These results may have been due to the decreased sensitivity of the early instrumentation used in these studies. Today, there is evidence that SYBR® Green I is suitable for use in HRM analysis for some applications, although it may be more difficult to optimize those reactions, and its success in HRM analysis may be instrument dependent [6].

Several different HRM dyes should be tested to determine which functions best in your experimental system. One factor affecting dye functionality is the instrument itself, since different instruments use different excitation sources and detection methods. Reagent choice along with characteristics of the amplicon may also influence the effectiveness of the dye.

Component	Volume (µL/rxn)
2X Master Mix	10
Magnesium Chloride Solution, 25 mM	variable
Forward Primer, 5 µM	1.2
Reverse Primer, 5 µM	1.2
SYTO® 9 Dye, 50 µM *	0.6
gDNA (5 ng/µL)	5
Deionized Water	variable
Total Reaction Mix Volume	20

*substitute other dye if desired

Table 2. Recommended PCR Setup for HRM Analysis.

2. HRM Assay and Reagent Optimization

Effects of $MgCl_2$ Concentration on HRM Analysis

The effect of $MgCl_2$ concentration on HRM analysis for detection of a single G/C point mutation is shown in Figure 10. Quadruplicates of each sequence variant were amplified in the presence of either 1 mM, 2 mM, 3 mM, or 4 mM $MgCl_2$. One representative of each homozygous variant G for each of the $MgCl_2$ concentrations was chosen as a control. The reference curve for the difference plot is a G control with 1 mM $MgCl_2$ (1 mM G). The autocalled group termed "Variant 1" contains C variants with 1 mM $MgCl_2$ concentration. The large gap between the two populations is ideal for easy discrimination of the two variant groups. At the 2 mM concentrations (2 mM G; all the G and C variants cluster together (dark blue lines). This clustering is also seen at 4 mM $MgCl_2$ concentrations (4 mM G; red lines). The 3 mM G (3 mM $MgCl_2$ concentration, G variant; purple and the light blue autocalled Variant 2 cluster that represents C variants at 3 mM $MgCl_2$) are distinctly separated into 2 variants, but the separation is much smaller than seen with the 1 mM G samples (dark green). This example clearly shows the effect of $MgCl_2$ on HRM results and highlights the need for a $MgCl_2$ titration when optimizing the PCR for HRM analysis.

Optimal annealing temperatures and cycle number for your system and reagents should also be determined. An optimized PCR will have a profile like that seen in Figure 11, and will display the properties described in the box, Ideal Amplification Properties for Successful HRM Analysis.

Whenever possible, samples with a known variant type should be prepared by the same procedure as unknown samples, and should be included on each PCR plate as a control.

Tip: Ideal Amplification Properties for Successful HRM Analysis

- Exponential phase of amplification is strong and steep
- C_T values of 20–25
- Reactions reach plateau
- Samples amplify similarly



Figure 11. Ideal Amplification Properties for Successful HRM Analysis.

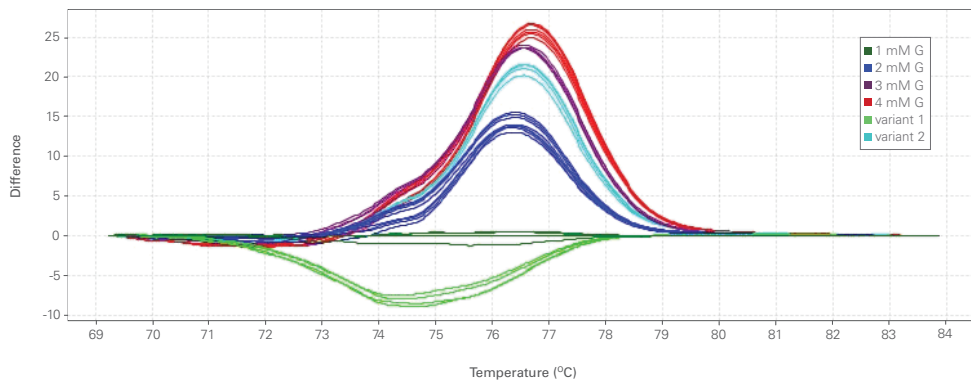


Figure 10. $MgCl_2$ Concentrations Affect HRM Analysis of a Single Point Mutation. Quadruplicates of each sequence variant were amplified in the presence of either 1 mM, 2 mM, 3 mM, or 4 mM $MgCl_2$ and the results were viewed as a difference plot.

2. HRM Assay and Reagent Optimization

Shopping List for HRM Analysis—Suggested Reagents			
Item	Size	Supplier	Part Number
Forward and reverse PCR primers (optional: HPLC purified)		Applied Biosystems	Custom
Appropriate reaction plate for your instrument			
MicroAmp® Optical 384-Well Reaction Plate with Barcode	0.1 mL, 50 plates	Applied Biosystems	4309849
	0.1 mL, 1,000 plates	Applied Biosystems	4343814
MicroAmp® Optical 96-Well Reaction Plate with Barcode	0.2 mL, 20 plates	Applied Biosystems	4306737
MicroAmp® Optical Adhesive Film		Applied Biosystems	4360954 4311971
AmpliTaq® 360 DNA Polymerase (includes AmpliTaq® 360 Buffer, MgCl ₂ , and optional GC enhancer)	250 U	Applied Biosystems	4398818
AmpliTaq Gold® 360 Master Mix	5 mL	Applied Biosystems	4398881
SYTO® 9 Green Fluorescent Nucleic Acid Stain or other suitable dye		Invitrogen	S-34854
Deionized water		Any reliable source	-
Pipettor & tips		Any reliable source	-
Microcentrifuge		Any reliable source	-

Tip: Optimizing PCR Reagents for Successful HRM Analysis

- Optimize the primer concentration—start at 300 nM
- Optimize the MgCl₂ concentration—start between 1.0–3.0 mM
- Use an AmpliTaq Gold® Enzyme optimized for PCR performance, such as AmpliTaq Gold® 360 Enzyme
- Test different primer pairs to determine which one is optimal
- Include proper controls for PCR and variants
- Include replicates for significance—triplicates are recommended

Tip: Other Suggestions for Successful HRM Analysis

- Use calibrated pipettes to ensure accurate pipetting
- Set up reactions on ice to minimize sample degradation and nonspecific reactivity
- Mix reagents well
- Keep reaction volumes at 20 µL or above, to minimize pipetting and mixing errors
- Be sure plates are well sealed
- Centrifuge before placing in the real-time instrument
- Run samples within 2 hr of preparing
- Prepare fresh master mix as needed

2.6 Instruments and Software

HRM Analysis Instrumentation

HRM analysis requires a PCR thermal cycler and an instrument with optics capable of collecting the numerous fluorescent data points; it is best when one instrument can perform both functions. Dual capability real-time PCR instruments are found in two designs:

- rotary**—samples spin past an optical detector
- block-based instruments**—samples are read by a scanning head or stationary camera

In block-based systems, a Peltier block is used for precision temperature control (see box, Real-Time PCR Systems for HRM Analysis). Using a real-time PCR system for both PCR and sample melt provides several advantages including:

- Walk away automation—no need to handle samples after loading
- Built in quality check—provides information on PCR efficiency, as well as detecting PCR failure or late C_T ; performing melt analysis on samples from poor PCR amplifications can produce spurious HRM results

Alternatively, PCR can be performed on a thermal cycler followed by transfer to a real-time instrument or another device for sample melt and data analysis.

HRM Analysis Software

Software for HRM analysis needs to be capable of handling the large amounts of data generated during an HRM experiment. Most HRM specific software use new algorithms and display plots which help discriminate DNA variants in HRM data. A good HRM software package should:

- Provide a view of the raw fluorescent melt data
- Provide a process to align that data
- View melt curve differences between samples

The Aligned Melt Curve is a typical starting view. The pre- and post-melt scaling regions are set within the software by the user or automatically through default software settings and define the data window used for this plot. Placement of the pre- and post-melt regions can affect resulting clusters and classifications of the HRM analysis results. The pre-melt region, in many instances, is the most critical, as correct placement of these bars can maximize differences between the variants, as characterized by their initial melt profile in the first stages of the active melt region. Care must be taken to ensure a region is chosen that is representative of the initial fluorescent levels. Usually this is quite close to the start of the active melt region, without actually encroaching on the melt curve itself. Viewing the melt data in the derivative view (Figure 12) can be useful when selecting the appropriate region for alignment.

Product: Real-Time PCR Systems for HRM Analysis

Applied Biosystems® 7500 Fast and 7900HT Fast Real-Time PCR Systems are ideal for HRM analysis applications. Both instruments:

- Enable real-time PCR and sample melt on a single instrument
- Maximize sensitivity to detect true variants and minimize false negative results

7500 Fast Real-Time PCR System

- Use of 'Expert Mode' to collect data in a single channel, maximizing the number of data points acquired during an HRM run
- Performs an optimized, short HRM melt window to decrease time-to-result
- Variable excitation capacity for optimum excitation of a full range of dyes

7900HT Fast Real-Time PCR Systems

Combines 96- and 384-well plate compatibility with fully automated robotic loading.

- Hands-free plate loading and unloading provides true walk-away automation allowing you to increase your lab's productivity
- Interchangeable blocks for flexibility in throughput

HRM Analysis Software

- Avoid the need for temperature shift correction, resulting in easy distinction between homozygous, mutant, and wild-type samples
- Shorten analysis time by autocalling genotypes and automatically omitting the no-template controls
- Minimize subjective analysis by automatically grouping unknown variant clusters

2. HRM Assay and Reagent Optimization

Applied Biosystems® HRM Software provides a variant or cluster result based on similarities of melt curve placement and shape. Controls can be used for comparison with experimental samples, providing detail on any differences present. When a sample clusters with a control, it is an indication that they are the same sequence. However, because HRM dyes bind nonspecifically, and different sequence variants can, at times, exhibit the same or very similar melt profiles, it is always good practice to confirm your HRM results with a more specific method such as with a TaqMan® Real-Time PCR Assay or by direct sequencing.

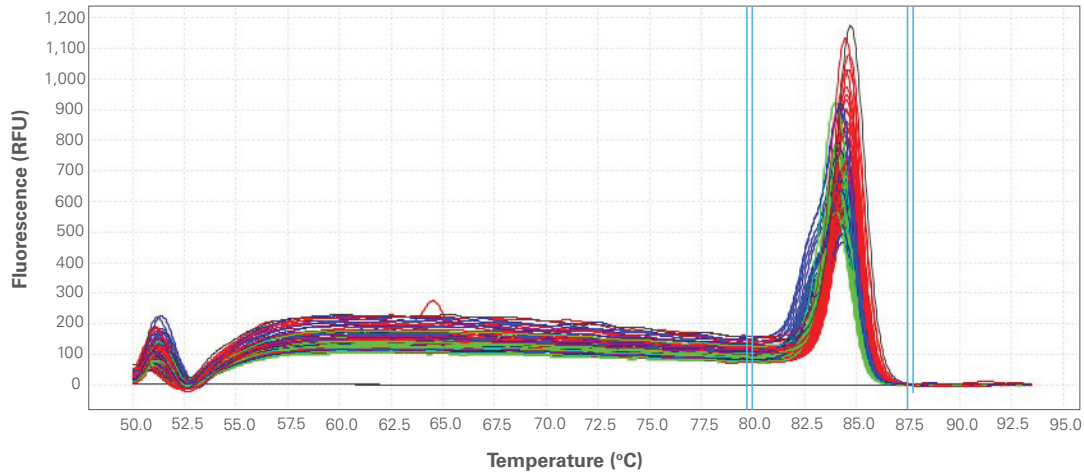


Figure 12. Using the Derivative View to Place Pre- and Post-melt Settings. This view can help ensure that the set pre- and post-melt regions do not encroach on the active region of the melt data. Colors represent different sample runs.

3. Troubleshooting Your HRM Experiment

3. Troubleshooting Your HRM Experiment

Problems with HRM experiments are usually evidenced by 1) abnormal PCR characteristics such as high C_t values or the presence of extra products, or 2) nonstandard melt data such as a wide spread in HRM curves from replicate samples or HRM curves with multiple melt domains. Table 3 identifies the source of such anomalous data and provides recommendations for resolution. Other Resources sidebar, *Other Troubleshooting Resources*, gives additional guidance.

Other Resources—Other Troubleshooting Resources

- **Real-time PCR On-line Troubleshooting Tool:**
www.appliedbiosystems.com/troubleshoot
- **HRM Getting Started Guide:**
http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_050347.pdf

	Observation	Issue	Effect on results	Try...	More Information
Aberrant PCR Results	C_t of majority of samples is >30	late amplification	reaction may not reach plateau and HRM resolution may be affected due to lower overall fluorescence gain	increase sample input or number of amplification cycles (beware of nonspecific amplification)	Review Chapter 2 (p. 8); especially 2.1: DNA Quality
	C_t of some samples >30	some late amplification	sample outliers with higher C_t than rest of group will have a T_m shift in HRM curve that may affect cluster calling	check relevant wells for volume discrepancies, look for signs of inhibition in amplification curve, increase sample input	Review Chapter 2 (p. 8) and see on-line PCR troubleshooting tool (listed in sidebar)
	amplification curves less steep and C_t values higher than expected	PCR inhibition	reaction may not be reaching plateau, effecting HRM resolution; HRM melt may also be inhibited	dilute DNA samples 1:10 or 1:100, or re-extract DNA from original sample material	Review Chapter 2 (p. 8) and see on-line PCR troubleshooting tool (listed in sidebar)
	presence of nonspecific products in PCR or larger than expected spread in HRM data	incorrect salt concentration	salt concentration affects PCR specificity as well as melting properties of amplicons	perform $MgCl_2$ titration to find best salt concentration for each reaction (note DNA eluate salt levels)	Review Chapter 2 (p. 8); especially 2.1: DNA Quality
	presence of extra melt curves (usually at lower temperature than expected product)	nonspecific products	PCR efficiency affected and presence of extra amplicons may impact melting behavior of true targets	ensure primer specificity with BLAST search; run product on gel to ensure single band; perform $MgCl_2$ titration	Review Chapter 2 (p. 8) and see on-line PCR troubleshooting tool (listed in sidebar)
Abnormal HRM Data	replicate samples show wide spread in HRM curves	replicates are widely spread	wide population spread leads to difficulty in assessing true sequence differences, particularly between two different homozygous populations	ensure appropriate salt concentrations, ensure similar DNA starting concentrations; ensure efficient PCR; consider using multiple controls for HRM analysis to assist in defining population spread	Review Chapter 2 (p. 8)
	complex melt curves with multiple melt domains	multiple melt regions	presence of more than one mutation site can result in complex melt curves that may be difficult to interpret; long sequences can also contain multiple melt regions in the absence of a SNP due to regional sequence context of amplicon	redesign primers; decrease amplicon size; sequence samples to confirm results	Review Chapter 2 (p. 8)
	multiple melt domains in HRM curve or greater than three distinct clusters, often with more than one heterozygous cluster	more than three clusters in genotyping experiment	presence of unknown SNPs in amplicon can create multiple heterozygous and homozygous amplicons; long sequences can also contain multiple melting regions in the absence of a SNP due to regional sequence context of amplicon	sequence product to confirm presence of additional SNP; redesign primers; reduce amplicon size	Review Chapter 2 (p. 8)
	very messy HRM curves usually present as diagonal wavy curves below heterozygous clusters	un-tasked NTCs	neglecting to omit negative controls and un-amplified samples from HRM analysis will skew pre- and post-melt settings and interfere with software cluster calls	test negative controls with instrument software and ensure negative results are omitted from HRM analysis	See the HRM Getting Started Guide (listed in sidebar)

Table 3. Troubleshooting Your HRM Experiment.

4. Summary

4. Summary

This guide has provided a starting point for performing robust HRM experiments. The most important take home message is the need to optimize the PCR step. Strong PCR will lead to HRM data that is easier to analyze, thus providing clearer results in which you can have more confidence. By optimizing sample quality, adjusting amplicon length for the desired application, confirming the specificity of primers, using the appropriate concentration of the chosen dye, defining the best reagent concentrations, and choosing an effective instrument and data analysis package, PCR will be more robust.

This guide has also addressed the usefulness of plotting data in different views. For example, how the derivative melt curve plot can be used to pinpoint an amplicon T_m , and how a difference plot can be used to accentuate differences between similar melt curves.

The next step is to apply the technology to your specific research question. The Applications Appendix on page 20 provides several specific examples for how this technology can be used for the identification of gene variants. See the sidebar, Other Resources, on p. 3 for additional resources.

5. Applications Appendix

Methylation-Sensitive High-Resolution Melting Analysis on 7500 Fast Real-Time PCR System

http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_053272.pdf

Summary: Step-by-step description of a workflow for the locus-specific analysis of DNA methylation by methylation-sensitive high resolution melting (MS-HRM) on the Applied Biosystems® 7500 Fast Real-Time PCR System.

Identifying Variants by High Resolution Melting (HRM) in Difficult Samples

<http://www.appliedbiosystems.com/HRM-FFPE>

Summary: An example of single nucleotide polymorphism (SNP) genotyping using an HRM technique on formalin-fixed, paraffin-embedded (FFPE) tissues. The HRM v2.0 Software was used along with the 7500 Fast Real-Time PCR System for genotyping of six DNA samples isolated from FFPE tissues. Results showed that the HRM Software used on the 7500 System can be an effective tool to screen for unknown SNPs across genes of interest in this difficult-to-use tissue.

6. References

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