A simple way to help strengthen your study

High-resolution melting for genotyping applications

What is HRM?

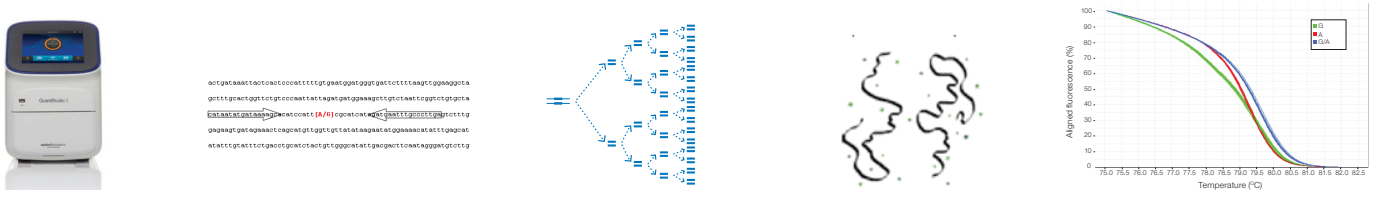
High-resolution melting (HRM) analysis is a post-PCR analysis method used for identifying genetic variation in nucleic acid sequences. This simple, fast method is based on PCR melting (dissociation) curve techniques and is enabled by the availability of improved double-stranded DNA (dsDNA)–binding dyes 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.

HRM analysis (Figure 1) starts with PCR amplification of the region of interest in the presence of a dsDNA-binding dye such as the SYTO™ 9 stain. This binding dye has 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 fluorescence 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.

How is HRM different from standard melt curve analysis?

HRM analysis differs from standard melt curve analysis (e.g., with SYBR™ Green dye) in three ways:

1. Chemistry—HRM analysis uses brighter dsDNA-binding dyes that do not inhibit PCR at high concentrations.
2. Instruments—HRM analysis requires instruments that collect more fluorescence data points, at finer temperature resolution.
3. Software—HRM analysis requires more sophisticated software that uses specific data analysis algorithms and plots.



Step 1: Instrument calibration	Step 2: PCR design and optimization	Step 3: PCR	Step 4: Sample melting	Step 5: Data analysis
MeltDoctor HRM Calibration Plate QuantStudio 3, QuantStudio 5, QuantStudio 6 Flex, QuantStudio 7 Flex, and QuantStudio 12K Flex Real-Time PCR Systems	Primer Express Software	MeltDoctor HRM Master Mix MeltDoctor HRM Reagent Kit MeltDoctor HRM Positive Control Kit QuantStudio 3, QuantStudio 5, QuantStudio 6 Flex, QuantStudio 7 Flex, and QuantStudio 12K Flex Real-Time PCR Systems	MeltDoctor HRM Master Mix MeltDoctor HRM Reagent Kit MeltDoctor HRM Positive Control Kit	High-Resolution Melt (HRM) Software Desktop or web browser–based software options available

Figure 1. HRM genotyping analysis workflow.

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HRM analysis also enables the determination of a more exact melting point of a DNA fragment and has significantly improved discrimination power to visualize differences between DNA fragment melt profiles.

Why use HRM in your genotyping project?

The melt profile of a sample reflects the mix of amplicons present. Characteristics such as GC content, length, sequence, and heterozygosity will contribute to the melt curve of each amplicon. The resulting profiles can provide valuable information for genotyping studies in addition to many other applications, including mutation screening and methylation analysis.

Applied Biosystems™ TaqMan™ SNP Genotyping Assays are currently the gold standard for accurate and reproducible PCR-based genotyping. TaqMan™ Assays are more sensitive and specific than HRM analysis, but HRM is well suited for some situations, including:

- Inability to design TaqMan™ probes due to inherent sequence limitations
- Analysis of a large number of single nucleotide polymorphisms (SNPs) with low sample numbers in cost-conscious settings
- Typing of highly mutable samples, where probe specificity may miss some species
- Analysis of nucleic acid variation in samples containing possible unknown SNPs
- Genotyping in nonmodel organisms, where sequence knowledge is low
- Association studies that require mapping of large regions
- Checking the validity of National Center for Biotechnology Information (NCBI)–derived SNPs in a project population

Factors to consider for HRM assay design

Primer design and PCR optimization

Efficient PCR amplification of the DNA region of interest is critical for successful downstream HRM analysis. Because the dyes used in HRM analysis bind nonspecifically to any double-stranded DNA products, PCR primers need to be designed for robust performance and must be specific to the region of interest. Many of the principles used in primer design for SYBR Green dye–based analysis should also be used for HRM analysis (Table 1). Applied Biosystems™ Primer Express™ Software is a primer design tool specifically designed for Applied Biosystems™ real-time PCR systems. Some assays may require the testing of several different primer pairs [1].

Amplicon length can also impact the sensitivity and specificity of HRM analysis. 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. In addition, as amplicon size increases, it is possible that additional SNPs or other mutations will be detected, which will result in a more complex melting pattern where it becomes more difficult to distinguish between the different sequence variants. Amplicons in the range of 70–130 bp are ideal for genotyping assays, and in many instances, 50–250 bp is also acceptable [1].

Primer concentration and purity should also be considered. Most assays are successful with primer concentrations of 300 nM, and concentrations can be increased up to 600 nM for troublesome assays. HPLC–purified primers are preferred, but may not be required for successful results.

Table 1. HRM PCR primer design guidelines.

Target sequence	<250 bp
Primer length	~20 nt
Primer melting temperature (T_m)	58–62°C, optimal 60°C
Forward and reverse primer T_m difference	<2°C
GC content	30–80%
GC clamp	Maximum of 2 G or C nucleotides in the last 5 nt at 3′ end
Avoid runs of identical nucleotides (especially G)	
Avoid complementarity within or between primers	
Avoid primers with homology to other targets	

Template quality

DNA template quality can greatly impact HRM results [1]. Salt carryover is one of the biggest factors impacting HRM results, because it can subtly change the thermodynamics of the DNA melting transition. Some commercial DNA isolation kits use high salt (1 M) for nucleic acid elution; 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.

Similar starting DNA concentrations should be used for all samples and controls in the HRM analysis. We recommend using high concentrations (10–20 ng of DNA per HRM reaction) for best results. For poor-quality nucleic acids, try increasing the starting DNA concentration or increasing the number of PCR cycles.

Inclusion of appropriate controls

HRM clustering accuracy can improve with the use of controls. For genotyping, use controls for each known variant within the test population—for example, the homozygote G/G, the homozygote A/A, and the heterozygote G/A. Test samples and controls should be of the same template type and at the same concentration. It is also important to use the same method to prepare DNA from all samples in an experiment, and that at least 3 replicates are performed for each sample.

Figure 2 illustrates the importance of assigning good controls for each genotype, helping to ensure accurate genotyping and potential mutation discovery.

The Applied Biosystems™ MeltDoctor™ HRM Positive Control Kit provides primers and control DNA for detection of a sample SNP mutation. This kit can be used for demonstration purposes and for troubleshooting of HRM analysis.

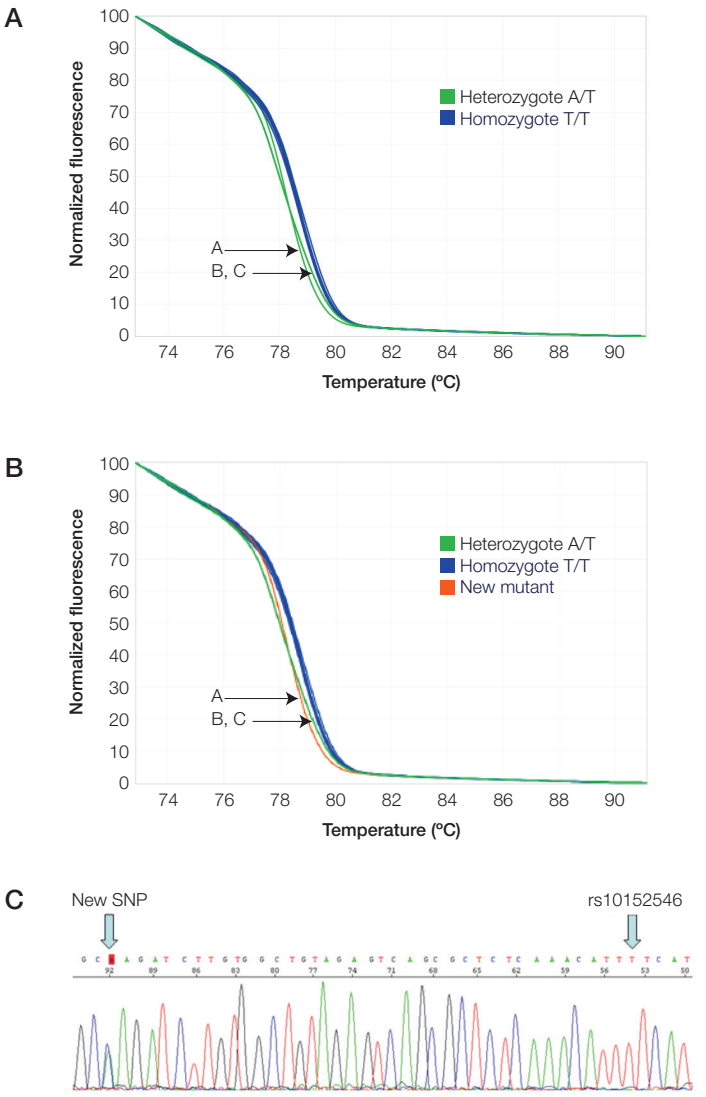

Figure 2. Inclusion of controls enables accurate HRM genotyping and mutation discovery. Coriell human gDNA samples were genotyped using a TaqMan SNP Genotyping Assay for SNP rs10152546 and subsequent HRM analysis. **(A)** Analysis without an assigned control. Samples B and C were genotyped as heterozygous (A/T) with both techniques. Sample A was genotyped as homozygous (T/T) with the TaqMan SNP Genotyping Assay and heterozygous (A/T) with HRM analysis. **(B)** After modification of the analysis parameters and assigning a control sample for samples B and C, the HRM Analysis Software identified sample A as a novel mutant. **(C)** Sequencing of sample A confirmed a T genotype for SNP rs10152546 and identified a new mutation 38 bp upstream of the SNP.

Table 2. Classes of single nucleotide polymorphisms (SNPs) in the human genome.

SNP class	Base change	Typical T_m curve shift	Occurrence in the human genome
1	C/T and G/A	Large (>0.5, avg. of 1.0°C)	64%
2	C/A and 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%

Quality of the HRM master mix

To minimize assay setup time and potential assay error, Applied Biosystems™ MeltDoctor™ HRM Master Mix is recommended for HRM genotyping analysis. This easy-to-use 2X formulation includes everything required for an HRM reaction, with the exception of primers and template. The master mix is formulated for excellent performance over a range of HRM assay types, including all SNP classes (Table 2), insertions and/or deletions, and methylation analysis. The master mix is stable for at least 3 months at 4°C after initial thawing.

Unlike some competitors' mixes, MeltDoctor HRM Master Mix was developed and optimized solely for HRM applications. The master mix includes Applied Biosystems™ AmpliTaq Gold™ DNA Polymerase, a highly purified hot-start DNA polymerase that minimizes nonspecific product formation and enables reactions to be set up at room temperature. Also included is the Applied Biosystems™ MeltDoctor™ HRM Dye, a stabilized form of the fluorescent SYTO 9 stain (for double-stranded nucleic acid) we developed. This dye possesses several significant properties important for high-performance HRM, including:

- Low background fluorescence
- High fluorescence in the presence of dsDNA
- Minimum temperature shift of DNA melting due to dye binding
- Thermal stability for tolerance of PCR cycling conditions
- No inhibition of polymerase activity, for high PCR efficiency

Comparison between HRM and TaqMan Assays for genotyping

TaqMan SNP Genotyping Assays use the 5´ nuclease assay for amplifying and detecting specific SNP alleles in purified genomic DNA samples. Each assay allows researchers to genotype individuals for a specific SNP.

With TaqMan Assays, a signal is generated by the SNP-specific probe; for example, the probe specific for an A allele generates a FAM™ dye signal, and the probe specific for a G allele generates a VIC™ dye signal.

Additional mutations between the probe and primer will not be detected with TaqMan Assays, and will have no impact on the signal generated during PCR. In contrast, any mutations between the two primers used in HRM analysis will generate variation in the resulting HRM profile. An example of such discrepant results between TaqMan Assays and HRM analysis is shown in Figure 3. In this example, the TaqMan Assay generates 3 genotypes, whereas HRM analysis generates 4 genotypes. There are two possibilities to explain this discordance:

1. The SNP is multi-allelic (e.g., A/G/C) instead of di-allelic (e.g., A/G). In this case, the third allele, C, cannot be detected by the TaqMan Assay because there are only 2 probes in the assay, one to detect A and one to detect G.
2. There is a second SNP in the PCR product. This second SNP may be either known or novel; checking a sequence database such as NCBI will confirm the presence of additional known SNPs. In the example shown in Figure 3, two additional SNPs were found close to the SNP studied (rs2875895): one 68 bases upstream and one 31 bases downstream. Sequencing of HRM PCR products confirms the presence and genotypes of each SNP.

Figure 3 demonstrates the need to check for the presence of SNPs around the region of a known SNP, before designing the HRM primers. Because TaqMan Assays detect only known SNPs, HRM analysis can be an effective method for detecting previously unknown SNPs.

Web browser–based HRM software for use with all QuantStudio Real-Time PCR Systems

Until recently, a different version of HRM software was needed to be compatible with the instrument platform used. With the release of the web browser–based software now available on Thermo Fisher Cloud, one software application will work across the Applied Biosystems™ QuantStudio™ family of real-time PCR systems. The HRM module is a browser–based application that can be run on any PC or Mac and does not require installation. Data can be accessed from any device with internet access and shared with collaborators at any time. The HRM application utilizes our latest analysis algorithm along with a modernized user interface. Up to 500 96-well or 384-well runs can be uploaded to a project and analyzed simultaneously, saving effort and analysis time.

Summary

HRM can be a powerful analysis tool for genotyping applications, particularly for screening experiments. The advantages of HRM include its low cost and ability to detect unknown genetic variants. However, in some cases the method can be less accurate than TaqMan Genotyping Assays, because some variant clusters may be difficult to distinguish.

There are instances when HRM genotyping assays will require optimization, including primer design and amplicon length. PCR conditions that can be optimized include increasing $MgCl_2$ concentration, increasing primer concentration (being careful to avoid primer dimerization), increasing extension time, and increasing PCR cycle number. Good laboratory practice is also important for successful HRM results. Techniques such as accurate pipetting and appropriate use of replicates and controls are essential.

Scientific contributors

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Reference

1. A Guide to High Resolution Melting (HRM) Analysis. Available for download at https://tools.thermofisher.com/content/sfs/manuals/cms_050347.pdf

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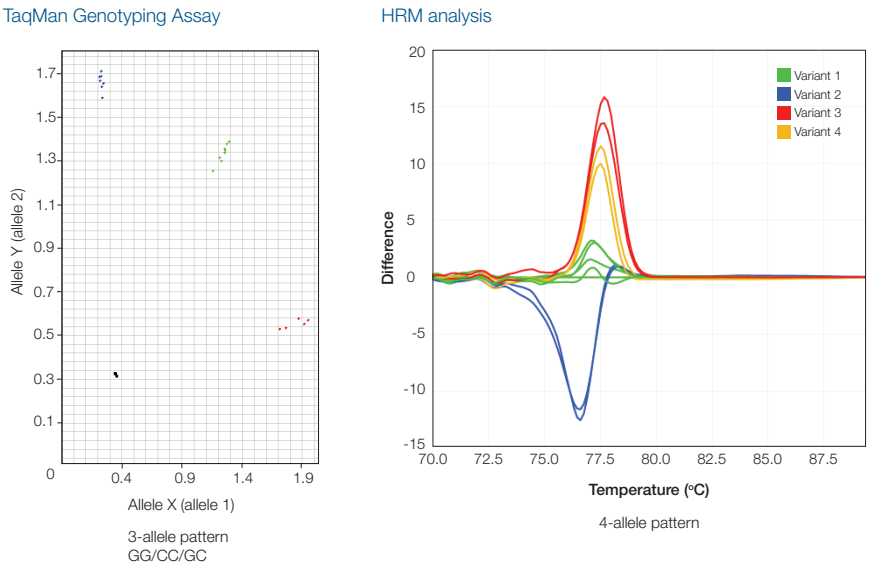

B

C

Group	rs2163038	rs2875895	rs2326280
1	G	G	A
2	G	G/C	A
3	G/C	G/C	A/T
4	G	C	A

Figure 3. Discrepant genotyping results between TaqMan Assays and HRM analysis. Coriell human gDNA samples were genotyped using a TaqMan SNP Genotyping Assay for SNP rs2875895, and subsequent HRM analysis. **(A)** The TaqMan analysis suggests the presence of 3 genotypes, whereas the HRM analysis indicates 4 genotypes. **(B)** The schematic illustrates the presence of additional SNPs distinct from that detected by the TaqMan probe, but within the region amplified for HRM analysis. Sequence analysis using the NCBI database identified the presence of two additional SNPs near the SNP detected by the TaqMan Assay (rs2163038 and rs2326280). **(C)** Sequencing of the HRM PCR products from each group validated the presence of all 3 SNPs, and determined the genotype for each.

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