Roche

Genetic Variation Analysis

Content

Introduction – Importance of Genetic Variation Research

Human beings show differences between their individual genomes on different levels: DNA sequences can carry single nucleotide polymorphisms (SNPs), insertions, deletions, or variations in the copy number of longer or shorter repeated elements. They can also vary in the way DNA is covalently modified, *e.g.*, by methlyation of CpG islands. Real-Time PCR offers different approaches to detect these variations.

Single nucleotide polymorphisms (SNPs) account for over 90% of all genome sequence differences between individuals. Many SNPs have no effect on cell function, but scientists believe others may predispose people to disease or influence drug response. In recent years, genotyping of SNPs has become a key component of genetic studies in fields, such as forensics, breeding of plants and animals, and especially pharmacogenomics.

Definition

SNPs and their classification

Single nucleotide polymorphisms, or SNPs (pronounced "snips"), are DNA sequence variations that occur when a single nucleotide (A,T,C or G) in the genome sequence is altered (see Fig. 1). For a variation to be considered a SNP, it must occur in at least 1% of the population. SNPs, which make up about 90% of all human genetic variation, occur every 100 to 300 bases along the 3-billion-base human genome. SNPs can occur in coding (gene) and noncoding regions of the genome. According to a widely accepted rule, SNPs can be classified as follows:

Class 1 SNPs are C/T and G/A transitions that produce C::G and A::T homoduplexes and C::A and T::G heteroduplexes.

In contrast, class 2 SNPs (C/A and G/T) are transversions that produce C::T and A::G heteroduplexes.

Class 3 SNPs (C/G) produce C::G homoduplexes with C::C and G::G heteroduplexes.

Class 4 SNPs (A/T) produce A::T homoduplexes with A::A and T::T heteroduplexes.

Of the possible single base pair polymorphisms, the A/T Class 4 SNPs are the most challenging to differentiate since homozygous genotypes differ least in their melting behavior. The typical difference in melting temperature is only about 0.2°C[3,6].

AAGCACGGATTCCA

AAGCACGAATTCCA

Figure 1   Single nucleotide polymorphisms.
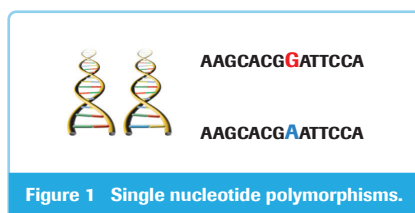
Before specific SNPs can be genotyped (*e.g.*, using sequencing or probe-based methods, see section 2.), whole genes or certain subfragments are often scanned to find out if previously unknown variations have arisen in a region of interest. PCR-based screening allows a reduction of sequencing sample number and cost because regions devoid of any unknown variations may be excluded from sequencing runs.

On the other hand, genotyping by real-time PCR is also frequently used to validate findings obtained previously on sequencing or microarray platforms, when moving from a whole-genome approach with few samples to a more limited set of targets and a higher number of samples.

Overview of Methods to Analyze Genetic Variation

In many genetic variation studies, large numbers of individuals must be genotyped. Alleles of known SNPs must be identified and labeled correctly and the presence of newly arising variants must be detected. The ideal genotyping method must be robust, quickly developed without extensive optimization, easy to use, automatable, and scalable.

With only a few exceptions, all current genotyping techniques start with a PCR amplification step. In most techniques, the first step is PCR amplification of a desired SNP-containing region, to increase assay specificity and sensitivity. After this first step, the method used depends on whether the aim is to discover unknown variants in the amplified region (mutation scanning), or to check for the presence of SNPs identified in similar setups (target regions in the samples of interest) in previous experiments (genotyping).

Gene Scanning versus Genotyping

- The basic, most commonly used method for detection of known variants is **Endpoint Genotyping** analysis, using enzymatically cleaved hydrolysis probes.

- A more advanced method is **Melting Curve Genotyping** analysis, using hybridizing (but not hydrolyzing) HybProbe or SimpleProbe probes.

- **Gene Scanning** is the discovery of new variants in target-gene derived amplicons. It can be easily performed based on the analysis of the melting behavior of these amplicons at high resolution, in the presence of a saturating DNA-binding dye.

TIP

When combined with DNA bisulfite modification protocols, high resolution melting approaches can be adapted to the study of methylation patterns (see Limitations and advantages of MS-HRM and bisulfite sequencing for single locus methylation studies[1]).

Detecting known variants → Specific Probes → Basic method: Endpoint Genotyping / Advanced method: Melting Curve Genotyping

Detecting any variation → High Resolution Melting Dye → Whole Amplicon Melting (*e.g.*, mutation scanning, methylation analysis)
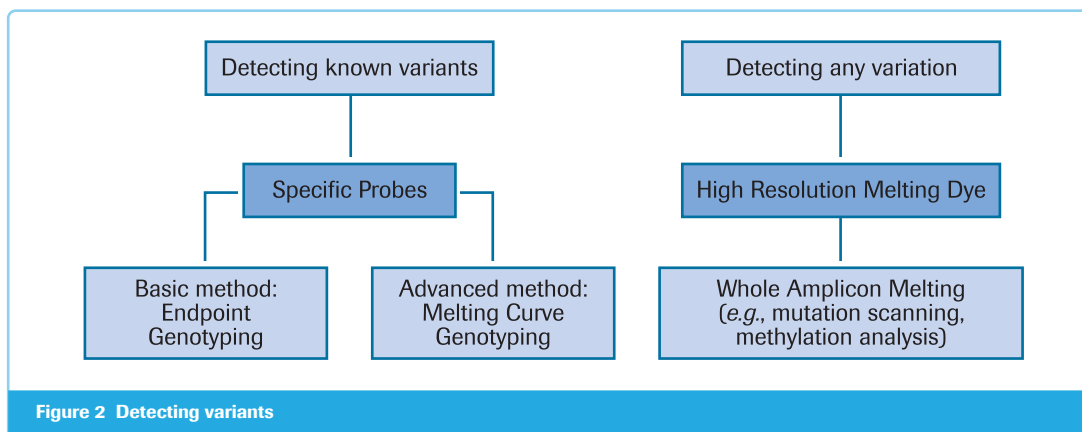
Figure 2 Detecting variants

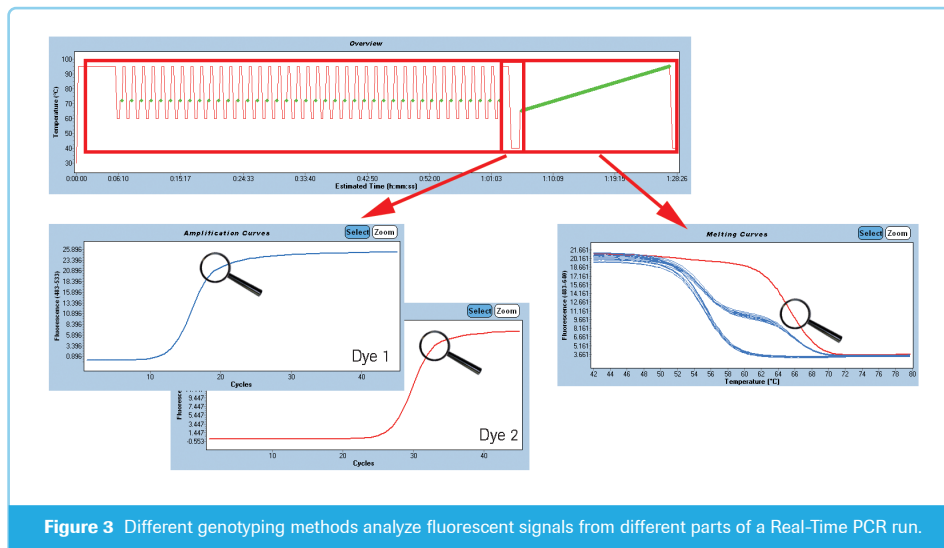# Overview of Methods to Analyze Genetic Variation

Overview of Different Methods for Real-time PCR Based Genotyping and Gene Scanning

Real-Time PCR Systems are characterized by their ability to display amplification curves that cover the entire PCR process. The curves result either from cleavage of a target-bound hydrolysis probe, or from the observed melting of a sequence-specific hybridization probe. According to the used detection format, different parts of the amplification process are exploited to reveal genotype information.

When allele-specific hydrolysis probes are used, the signal generated by their cleavage will accumulate during amplification, and its endpoint value may be used to determine the genotype(s) present (see Fig. 3. left).

In contrast to that, melting curve analysis is performed post-PCR, with generated data providing additional information about the melting behavior of the product, facilitating the identification of a specific SNP allele or allele combinations (see Fig. 3, right).

Details for both approaches are explained in the next section.

Figure 3 Different genotyping methods analyze fluorescent signals from different parts of a Real-Time PCR run.

Detecting Known Variants

1. Basic Method: Endpoint Genotyping

Endpoint Genotyping assays use hydrolysis probes (for an explanation of this probe format see Appendix 1). Each probe contains two labels, a fluorescent reporter and a quencher, in close proximity to each other. Endpoint Genotyping analysis uses two sequence-specific probes that are designed to detect allele X and allele Y and are labeled with different reporter dyes(see Fig. 4).

Standard Set-up
- FAM dye detects samples that are homozygous for allele X.
- VIC/HEX dye detects samples that are homozygous for allele Y.

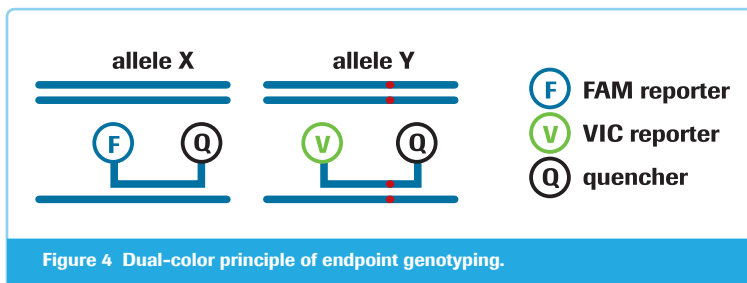### Endpoint genotyping is based on a dual color approach.

Figure 4 Dual-color principle of endpoint genotyping.

Data are collected throughout the PCR amplification; however, only the endpoint signal intensities of the two reporter dyes are used to identify the genotypes. The relative dye intensities can readily be visualized on a scatter plot, simplifying the discrimination of homozygous X, homozygous Y, and heterozygous samples(see Fig. 5).
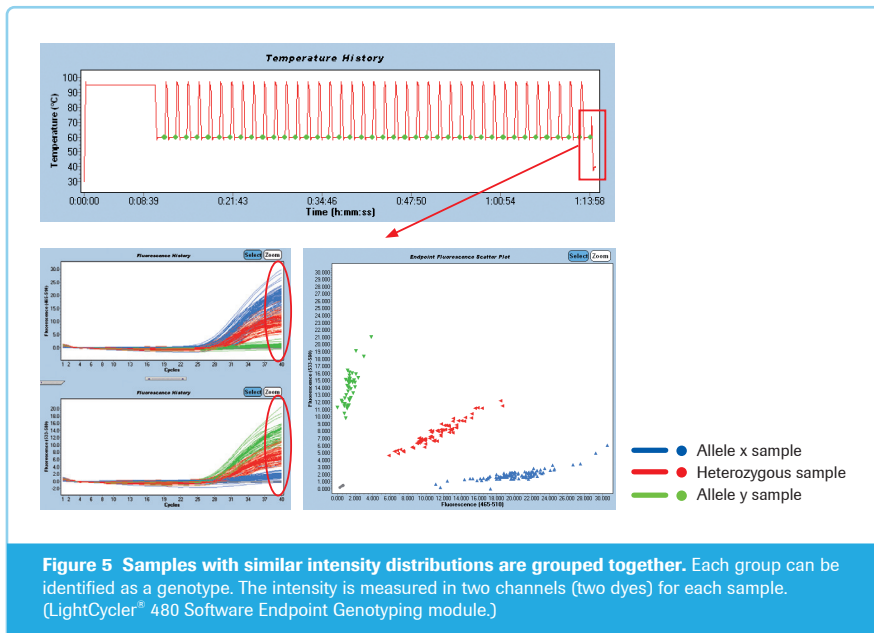

Figure 5 Samples with similar intensity distributions are grouped together. Each group can be identified as a genotype. The intensity is measured in two channels (two dyes) for each sample. (LightCycler® 480 Software Endpoint Genotyping module.)

TIP

For endpoint genotyping, use reagents specifically designed for hydrolysis probe detection. The LightCycler® 480 Probes Master, a ready-to-use reaction mix is the ideal choice for hydrolysis probe detection assays in multiwell plates on the LightCycler® 480 Instrument.
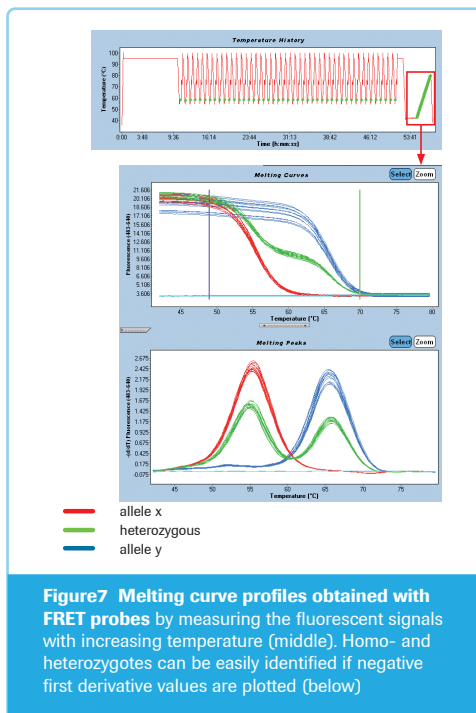
2. Advanced Method: Melting Curve Genotyping

Melting curve analysis using HybProbe or SimpleProbe methodology is a reliable means for studying known variations. Allele-specific primers or probes are not required; the same pair of probes (anchor and sensor probes) binding next to each other on the target region is used for all alleles (generally two, but sometimes more) of one given SNP.

Mismatch

Perfect Match

Temperature

Low   Medium

Figure 6   Principle of melting curve genotyping.
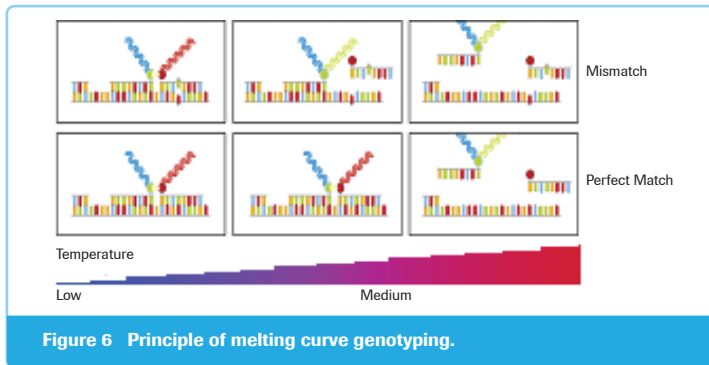
As the sequence-specific anchor and sensor probes bind next to each other on the target DNA, fluorescence signal is generated via the FRET (fluorescence resonance energy transfer) process. Since the temperature increases during melting curve analysis, the fluorescence signal decreases as the probes melt away from the template DNA. A single base change occurring within the region recognized by the probe will lead to thermal destabilization of the probe(s)-target complex. Thus, the melting temperatures (Tms) will be different for amplicons with sequence differences (SNP alleles). Therefore, genotyping information can be derived from the shape of the observed melting curve(see Fig. 7).

allele x
heterozygous
allele y

Figure7   Melting curve profiles obtained with FRET probes by measuring the fluorescent signals with increasing temperature (middle). Homo- and heterozygotes can be easily identified if negative first derivative values are plotted (below)

TIP

The LightCycler® 480 Genotyping Master is designed for Real-Time PCR, followed by melting curve analysis on the LightCycler® 480 Instrument. It can be used to genotype single nucleotide polymorphisms (SNPs) and to analyze mutations. It is especially recommended for multiplex approaches. Please note that this Master mix is not suitable for endpoint genotyping with hydrolysis probes, as it contains a 5'-3'-exo-minus Taq DNA Polymerase.

3. Pros and Cons of Basic and Advanced Methods

Endpoint genotyping is a basic method that can be set up and performed quickly without optimization. Assays are available from different providers, thus, assay design is not a concern of the user. For many targets, ready-to-use primers and probes are commercially available and can be used under standard PCR conditions. Endpoint genotyping with hydrolysis probes has therefore become a widely used, basic approach; the all-in-one reaction mixes are easy to use and results can be interpreted straightaway.

However, usually only one mutation per assay can be investigated, *i.e.*, the technique is not suitable for haplotyping (studying combinations of neighboring SNPs that are covered by the same probe) or SNPs that have more than two different alleles. The method also generally fails in the presence of new, unexpected mutations. Last but not least, since this method requires a biochemical reaction and is not just biophysical as is melting curve analysis, it may be prone to disturbances caused by experimental conditions (*e.g.*, impure sample preparation) under which the hydrolysis reaction is not optimal.

The more advanced and flexible melting curve genotyping provides more insights into complex genetic constellations than basic hydrolysis probe chemistry. It even allows analysis of several variable sites in combination (*e.g.*, haplotypes). When used in multiplex assays, this technique can detect multiple SNPs. The superiority of this method to detect new mutations has been described by different authors[8,14].

On the other hand, melting curve analysis requires careful probe design to ensure that the probe sequence covers at least one SNP, and optimization of each assay. Software solutions (*e.g.*, LightCycler® Probe Design Software2.0) and commercial services (listed on www.lightcycler480.com) are available to support researchers who are new to this method.

Detecting Unknown Variants by High Resolution Melting (HRM)

1. Principle and Definitions: Gene Scanning by High Resolution Melting

Information on the presence of unknown mutations can be derived from melting curve data that are collected in the absence of a specific probe, at high resolution after PCR. High Resolution Melting (HRM) requires only PCR reagents, a simple primer pair for amplifying the gene of interest, a saturating DNA-binding dye, and an instrument with precise temperature control. The LightCycler® 480 Real-Time PCR System´s hardware, software and reagents have been designed to optimally meet these requirements.

When DNA is melted and allowed to slowly reanneal after PCR, the presence of a SNP (heterozygous in most cases) will generally result in homo- and heteroduplexes of both strands. Heteroduplex DNA melts at a different tempera-ture than homoduplex DNA, because of a sequence mismatch between hybridized strands. This difference in melting temperatures can be detected if a saturating DNA-binding dye, such as LightCycler® 480 ResoLight is used (Fig. 9 and 10). High Resolution Melting dyes can detect the presence of heteroduplexes formed during PCR (*e.g.*, if the sample is heterozygous for a particular mutation). The possible applications exceed those of other, more traditional DNA-staining dyes such as SYBR Green I. Since it is not toxic to amplification enzymes, high concentra-tions of the dye do not affect the PCR step itself. These high concentrations completely saturate the dsDNA in the sample. Therefore, when dye molecules dissociate from dsDNA during melting, there is only a slight chance for them to re-bind to other unoccupied sites. This makes the melting process highly homogeneous and the acquired signals very sharp. Under these conditions, even small changes in the melting curve result in subtle, but reproduc-ible changes in fluorescence.

The melting curve data, if displayed on a difference plot, will identify clusters of the samples (*e.g.*, homozygous wild type, homozygous mutant, and heterozygous Fig. 8).
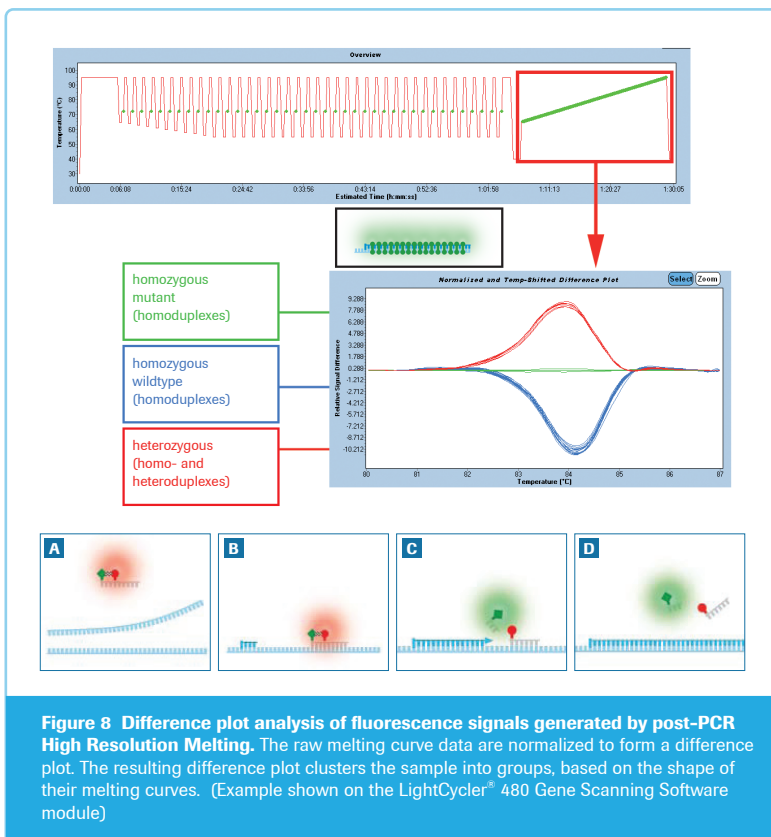

Figure 8 Difference plot analysis of fluorescence signals generated by post-PCR High Resolution Melting. The raw melting curve data are normalized to form a difference plot. The resulting difference plot clusters the sample into groups, based on the shape of their melting curves. (Example shown on the LightCycler® 480 Gene Scanning Software module)

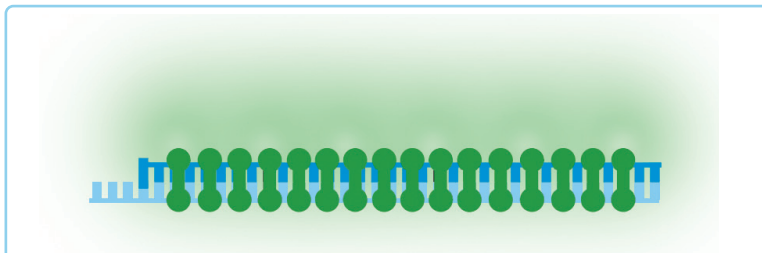# Detecting Unknown Variants by High Resolution Melting (HRM)

Figure 9 Saturating dye bound to double-stranded DNA. Saturation of the amplicon leaves no room for relocation events during melting and allows sharp distinction of different DNA duplexes.
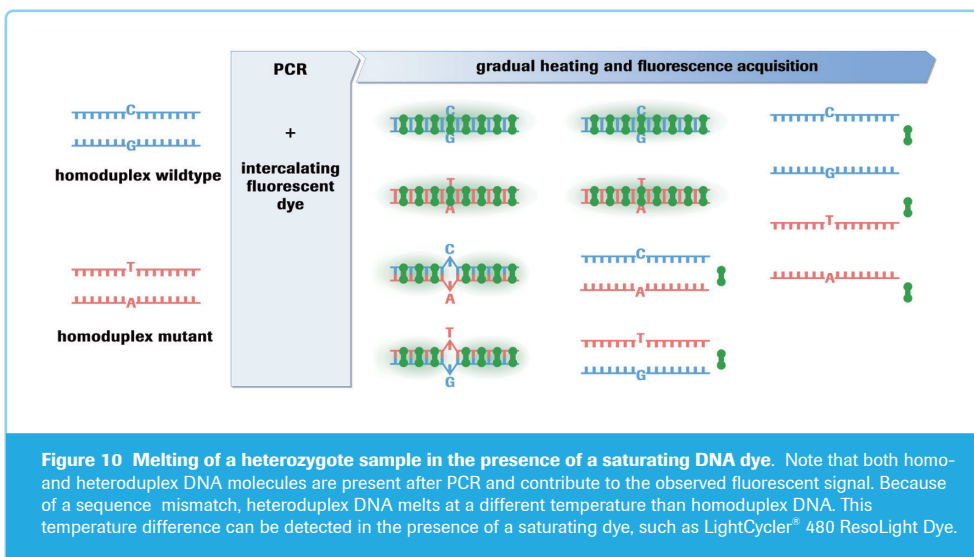

Figure 10 Melting of a heterozygote sample in the presence of a saturating DNA dye. Note that both homo- and heteroduplex DNA molecules are present after PCR and contribute to the observed fluorescent signal. Because of a sequence mismatch, heteroduplex DNA melts at a different temperature than homoduplex DNA. This temperature difference can be detected in the presence of a saturating dye, such as LightCycler® 480 ResoLight Dye.

2. Optimizing HRM Assays

To ensure an effective gene scanning assay, the experiment must be designed carefully.

We recommend the following steps when planning and performing high resolution melting experiment:

- Carefully design the experiment
- Choose the best primers
- Standardize sample preparation
- Optimize the reaction mixture
- Optimize the PCR and melting programs
- Analyze the experimental data with appropriate software
- Use additional tools and downstream techniques for more detailed information

Detecting Unknown Variants by High Resolution Melting (HRM)

2.1 Carefully Design the Experiment

If the exon to be scanned is not too long (maximum length 400 bp, ideally < 250 bp) place the primers in the adjacent introns. This allows the entire exon to be scanned as a single amplicon.

Longer exons should be divided into several segments (*e.g.*, amplicons of approx. 300 bp each). Design the primers for these segments in a way that the amplicons overlap.

If you want to analyze only certain hot spots or sites with a known polymorphism, short amplicons (< 150 bp) are preferable. A single base variation affects the melting behavior of a 100 bp amplicon more than that of a 500 bp amplicon; thus, short amplicons are more likely to show the effects of small sequence variations.

It is possible to detect sequence variations with longer amplicons. Nevertheless, the influence of a variation on the melting curve shape decreases with an increasing amplicon length and amplicons > 500 bp often exhibit a multiphase melting behavior, disturbing the variation detection (more than 2 melting domains will preclude proper analysis).

2.2 Choose the Best Primers

Amplicons contaminated with artifacts, such as primer dimers or nonspecific products, can make results difficult to interpret. Therefore, to obtain the best gene scanning results, design your primers carefully. Accurate primer the design ensures specific primer binding at the desired target sequence and minimizes the formation of primer dimers. Primers should not bind at positions that have secondary structure.

TIP

> Always use highly purified (*e.g.*, HPLC purified) primers and low primer concentrations (*e.g.*, 200 nM each) to avoid primer dimer formation.

To obtain the best HRM results, not all pre-developed and published primers are optimally suited. Sometimes the design of new primers and the optimization according to the prerequisites of real-time PCR is recommended to generate better results faster.

TIP

> Parallel testing of more than one set of primers will increase the likelihood of quick access to gene scanning.
>
> Follow these general guidelines:
>
> - Use special software to design the primers, *e.g.*, Primer3
> - Design PCR primers that have annealing temperatures around 60°C.
> - Avoid sequences that are likely to form primer dimers or nonspecific products.
> - BLAST (http://www.ncbi.nlm.nih.gov/BLAST) the primer sequences, to ensure they are specific for the target species and gene.
> - Always use primers that have been purified by HPLC.
> - Use low primer concentrations (*e.g.*, 200 nM each) to avoid formation of nonspecific products, such as primer dimers.
> - Check the specificity of the PCR product (*e.g.*, on an agarose gel). Remember that reactions containing primer dimers or nonspecific products are not suitable for HRM analysis.

Tools to predict a secondary structure, such as mFOLD are available and may be used to analyze the sequence of interest. Please verify that the conditions used for the *in silico* analysis follow the conditions used within the PCR reaction.

2.3 Standardize Sample Preparation

Since gene scanning analysis compares amplicons from independent PCRs, reaction-to-reaction variability must be minimized. One way to achieve this is to standardize the sample preparation procedure.

TIP

> For optimal HRM analysis, all amplification curves should have a crossing point < 30. More importantly, check that all curves reach similar plateaus. Salts affect DNA melting behavior, so ensure that the concentrations of buffer, Mg^{2+} and other salts in the reaction are as uniform as possible for all samples.

HRM compares amplicons from independent PCR reactions; therefore minimizing reaction-to-reaction variability is essential. One way to minimize variability is to standardize the sample preparation procedure.

TIP

> - Use the same extraction procedure for all samples.
>
> - Use nucleic acid preparation techniques that are highly reproducible. For example, you could use one of the following:
>   - one of the MagNA Pure LC Instruments, or the MagNA Pure 96 Instrument, or the MagNA Pure Compact Instrument (see www.magnapure.com) together with together with a dedicated nucleic acid isolation kit (for automated isolation), or
>   - a High Pure nucleic acid isolation kit (for manual isolation), *e.g.*, the High Pure PCR Template Preparation Kit.
>
> - Determine the concentration of the DNA samples using spectrophotometry, then adjust samples to the same concentration with the resuspension buffer.
>
> - Use the same amount of template in each reaction (5 to 30 ng template DNA in a 20 µl reaction).
>
> - Check the Cp values and the height of the amplification curves for all samples
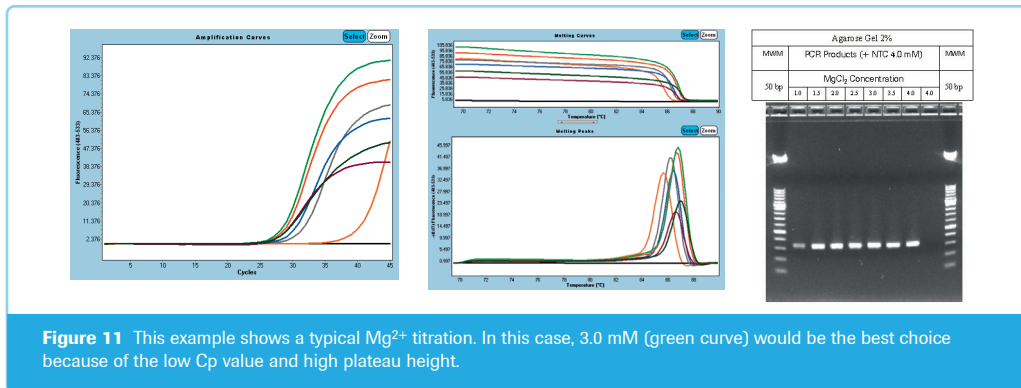
2.4 Optimize the Reaction Mixture

Salts affect DNA melting behavior, so it is important that the concentrations of buffer, Mg2+ and other salts in the reaction mix are as uniform as possible across all samples.

To ensure both the specificity and robustness of the PCR, always determine the optimal MgCl$_2$ concentration for each experimental system. We recommend that you titrate the MgCl$_2$ concentration in the reaction between 1.5 and 3.5 mM (in 0.5 mM steps) when establishing a new assay (Fig. 11).

You can verify the quality of the amplicons on an agarose gel or by standard melting curve analysis.

Optimization can be simplified by using the LightCycler® 480 High Resolution Melting Master, a ready-to-use, hot-start reaction mix that is designed to produce optimal HRM results on the LightCycler® 480 Instrument.

Figure 11 This example shows a typical Mg^{2+} titration. In this case, 3.0 mM (green curve) would be the best choice because of the low Cp value and high plateau height.

2.5 Optimize the PCR and Melting Programs

PCR program:

Annealing temperature is the thermal cycling variable with the greatest influence on PCR specificity and robustness.

TIP

If you do not know the actual melting temperatures of your PCR primers (the calculated melting temperatures are often not accurate, so do not rely too heavily on them), it is best to use a touchdown PCR protocol that covers a range of annealing temperature between 65 and 55°C

With touchdown PCR, a relatively high annealing temperature is used in the early cycles of PCR, to ensure high accuracy of priming and amplification. Decreasing the annealing temperature in later cycles ensures that adequate amounts of PCR product are finally obtained (see Fig. 12).

Figure 12 This is an example of a touchdown PCR program and profile.
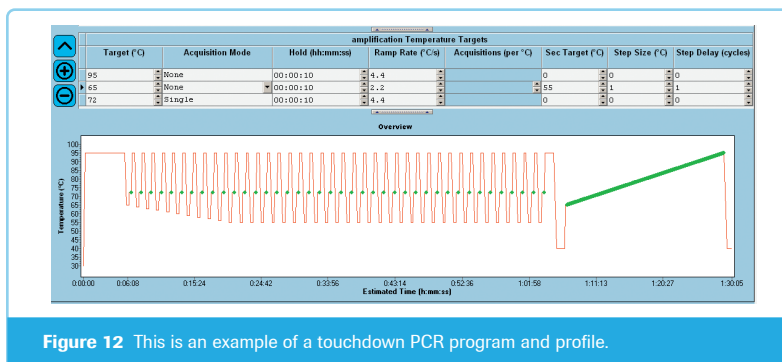
Melting program:

Gene Scanning experiments are performed in the melting curve analysis mode. When setting up an HRM protocol, the following points should be considered:

Rapidly cooling the mixture to 40 °C encourages heteroduplex formation and ensures that all PCR products have re-associated.

Actual melting conditions depend upon the amplicon. For initial experiments, set a wide melting interval, *e.g.,* 60 to 95°C. Once you have determined the actual temperature at which the product will melt, you should reduce the melting interval to a maximum of 25°C. However, you should ensure that the melting program starts at least 10°C before and ends at least 5°C after the expected Tm value.

Twenty-five acquisitions per °C are sufficient to result in a resolution appropriate for HRM analysis.

high resolution melting Temperature Targets							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
95	None	00:01:00	4.4				
40	None	00:01:00	2.2				
75	None	00:00:01	1				
90	Continuous		0.02	25			

Figure 13 This example shows a typical High Resolution Melting protocol.

TIP

Allow sufficient data collection time for pre- and post-melt phases. Capture HRM data points within a range of approx. 10°C (or greater), centered around the observed melting temperature. This provides enough baseline data points for effective curve normalization and will result in better replicates and easier data interpretation.
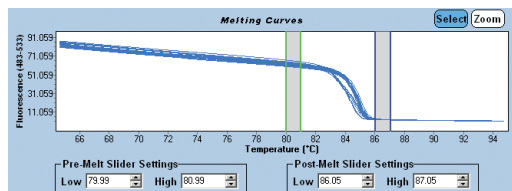

Figure 14 Correct setting of pre- and post-melt sliders is key for clear HRM results.

Negative, or no template controls (NTC) should be included in every HRM run. If available, controls that contain the wild-type and/or the mutant genotype should be included in the run to verify the results.

2.6 Analyze the Experimental Data with Appropriate Software
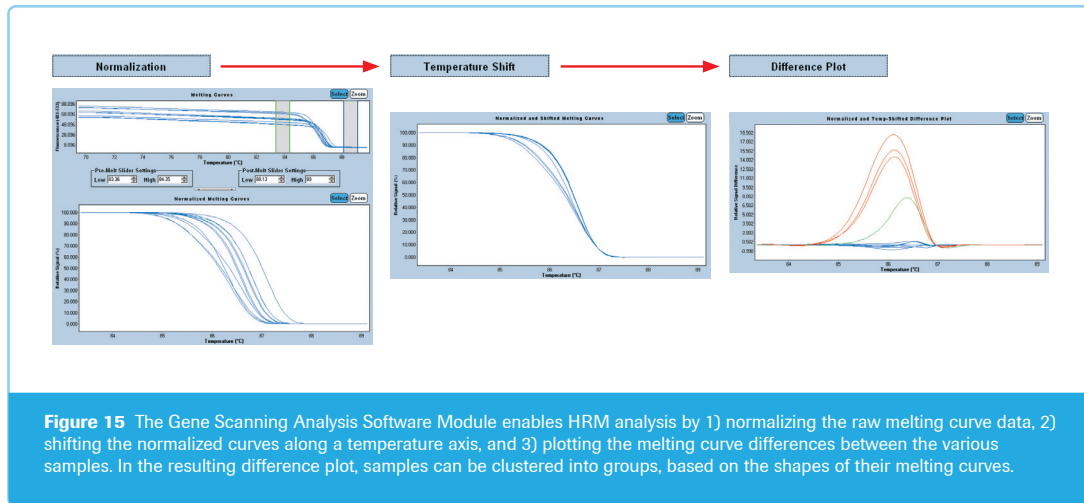
Prior to high resolution melting analysis, always review amplification data first. In general, after a steep initial rise, the signal should reach a plateau at > 30 relative fluorescence units. A crossing point below 30 cycles shows an adequate amount of sample material and a suitable amplification efficiency. Between samples, the crossing point should not vary more than 5 Cp units (corresponding to approximately a 1:100 dilution).

Because of the possibility to monitor the performance of the amplification, a workflow on a real-time PCR instrument should always be preferred over block cycler amplification.

The LightCycler® 480 Gene Scanning Software normalizes the raw melting curve data (Fig. 15) by setting pre-melt (initial fluorescence) and post-melt (final fluorescence) signals of all samples to uniform values. In the next step (temperature shift, Fig. 15), the software shifts the normalized curves along the temperature axis, to equalize the point at which the dsDNA in each sample becomes completely denatured.

The final step (difference plot, Fig. 15) involves subtracting the shifted, normalized curves from a reference curve (also called "base curve") to obtain a clearer display of the differences in melting curve shape. In the resulting difference plot, samples can be clustered into groups of similar melting curve shape.

Detecting Unknown Variants by High Resolution Melting (HRM)

Figure 15 The Gene Scanning Analysis Software Module enables HRM analysis by 1) normalizing the raw melting curve data, 2) shifting the normalized curves along a temperature axis, and 3) plotting the melting curve differences between the various samples. In the resulting difference plot, samples can be clustered into groups, based on the shapes of their melting curves.

If the difference plot does not give satisfactory results, the raw melting curves should be checked for evidence of primer dimers or nonspecific by-products created during amplification.

TIP

Software can streamline processes and increase speed and convenience, as well as data quality.

To speed up the process of setting up an experiment, use a software that contains templates for all genetic variation analysis methods described in this note (Endpoint Genotyping, Melting Curve Genotyping, Gene Scanning).

Software modules that combine and compare analysis results previously generated, or create studies that summarize the results from multiple experiments, add convenience to projects on a larger scale. The LightCycler® 480 Multiple Plate Analysis Software is recommended to perform this task.
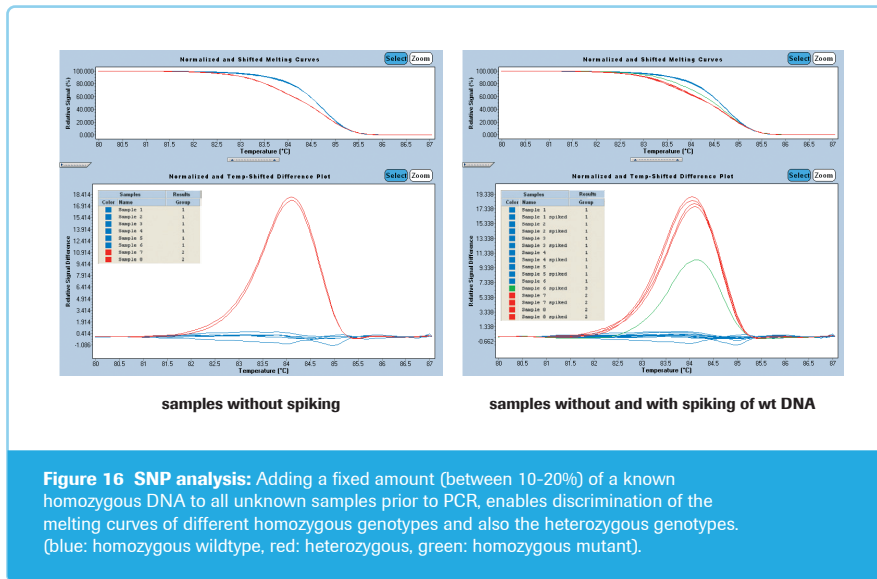
3. Advanced HRM Applications and Downstream Techniques

3.1 Differentiating Homozygous Samples

When the amplicons from heterozygous samples (containing both homo- and heteroduplexes) are melted, the resulting curves have a characteristic shape, different from the typical shape that results from a homozygous sample. Depending on the individual sequences, amplicons from different homozygous variants sometimes generate similarly shaped curves that are hard to distinguish.

In some cases, homozygous variants cannot be distinguished from each other when used in an unmodified form. Spiking all samples with a known amount of wild-type DNA offers a solution in cases such as this, thus ensuring clear differentiation of homozygous variants (Fig. 16).

samples without spiking | samples without and with spiking of wt DNA

Figure 16 SNP analysis: Adding a fixed amount (between 10-20%) of a known homozygous DNA to all unknown samples prior to PCR, enables discrimination of the melting curves of different homozygous genotypes and also the heterozygous genotypes. (blue: homozygous wildtype, red: heterozygous, green: homozygous mutant).

3.2 Using DNA Sequencing to Type Variations

Gene Scanning can detect any sequence variation compared to other samples, but it does not determine exactly which base is present in the respective alleles. Therefore, most researchers rely on sequencing one amplicon of each group to define the variations.

TIP

Ensure that the dye used during real-time PCR does not interfere with the sequencing process. With LightCycler® 480 ResoLight Dye, amplicons can be directly used in the sequencing reaction. However, prior to the sequencing reaction, we recommend to remove dNTPs and primers from the amplicon mixture (*e.g.*, using the High Pure PCR Product Purification Kit).

Beyond the scope of Real-Time PCR, Roche Applied Science also offers next-generation sequencing and microarray systems, enabling you to answer a broad range of questions related to genetic and genomic variation (visit www.454.com and www.nimblegen.com for more information).

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