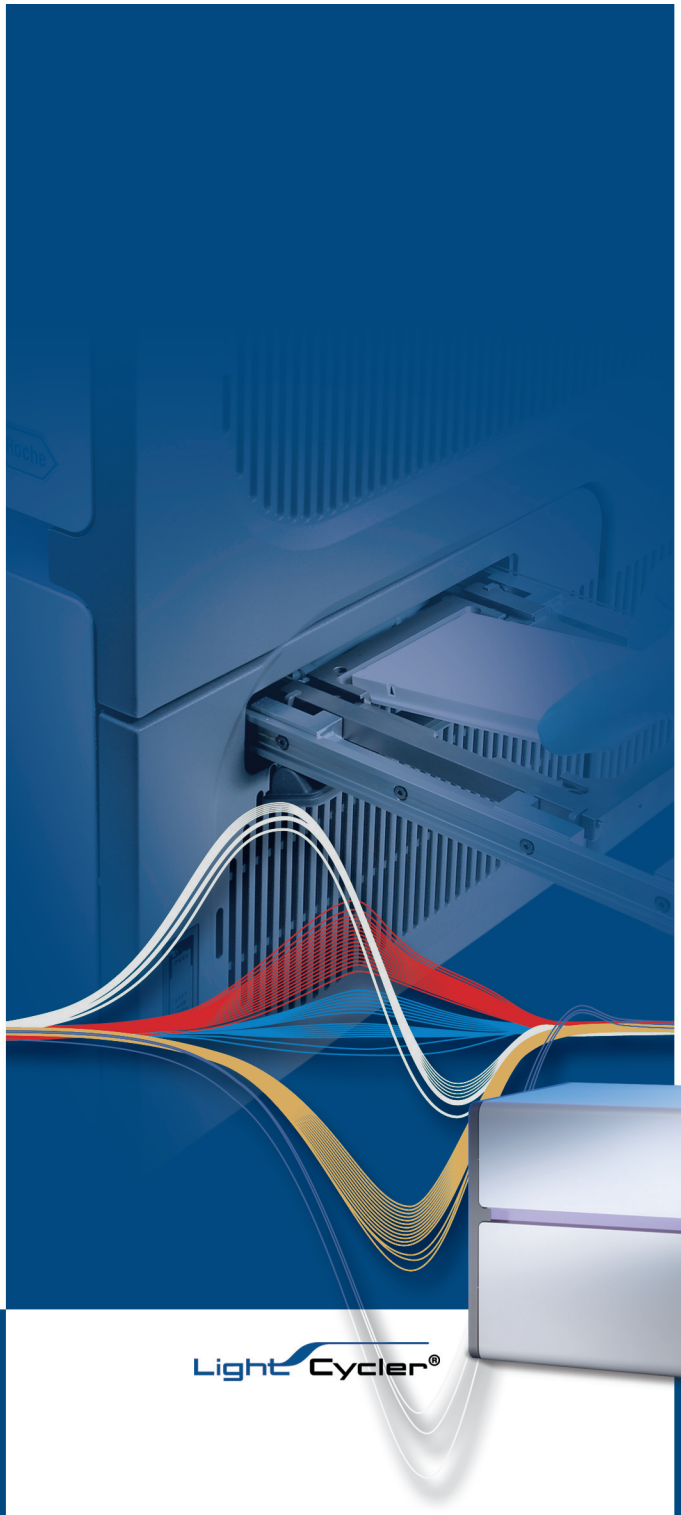
LightCycler® 480 Real-Time PCR System

Technical Note No. 1



High Resolution Melting: Optimization Strategies

High resolution melting (HRM) is a novel, closed-tube, post-PCR technique allowing genomic researchers to easily analyze genetic variations in PCR amplicons.

This technical note describes general steps of setting up HRM-based PCR assays, with a special focus on ways how to optimize procedures for gene scanning experiments.

High Resolution Melting: Optimization Strategies

Introduction

The novel High Resolution Melting (HRM) technique enables researchers to rapidly and efficiently discover genetic variations (*e.g.*, SNPs, mutations, methylations). HRM provides outstanding specificity and sensitivity with high sample throughput. Using the LightCycler® 480 Real-Time PCR System, PCR and analysis can be performed on one instrument, which is convenient, saves time and reduces costs.

Currently, the main application for the High Resolution Melting method is gene scanning, *i.e.*, the identification of heterozygotes in order to discover new variations in target-gene-derived PCR amplicons (Figure 1).

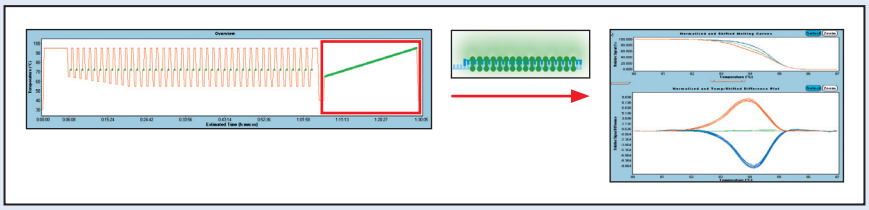


Figure 1: High Resolution Melting is a refinement of melting curve analysis that uses a saturating DNA-binding dye. After the PCR amplification and subsequent melting under high resolution conditions, the Gene Scanning Module in the LightCycler® 480 Software processes the raw melting curve data to form a difference plot. This method can reveal variations between samples (*e.g.*, mutations or DNA modifications) and display those variations on a graph that is easy to interpret.

Overview of High Resolution Melting Principle

In High Resolution Melting experiments, the target sequence is amplified by PCR in the presence of a saturating fluorescent dye (*e.g.*, LightCycler® 480 ResoLight Dye) (Figure 2).

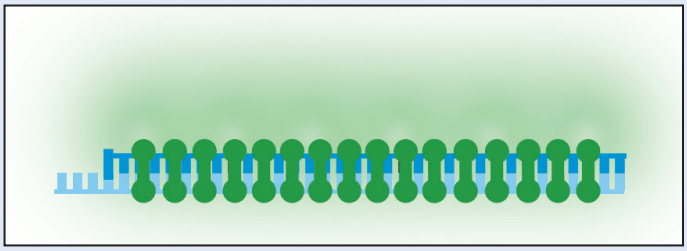


Figure 2: LightCycler® 480 ResoLight Dye bound to double-stranded DNA. Saturation of the amplicon leaves no room for relocation events during melting.

As with any dye used in melting experiments, the HRM dye fluoresces strongly only when bound to dsDNA. This change of fluorescence during an experiment can be used both to measure the increase in DNA concentration during PCR amplification and, subsequently, to measure temperature-induced DNA dissociation during High Resolution Melting.

After PCR in the presence of the dsDNA-binding fluorescent dye, amplicons are briefly denatured and then rapidly reannealed. If the DNA sample is heterozygous, perfectly matched hybrids (homoduplexes) and mismatched hybrids (heteroduplexes) are formed (Figure 3).

When the temperature is slowly increased again, the DNA begins to melt. Fluorescent signal from a heterozygous DNA sample shows a decrease at two characteristic temperatures, due to the different rates of strand separation in heteroduplex and homoduplex dsDNA (Figure 3). Thus, the shapes of

Overview of High Resolution Melting Principle continued

melting curves obtained with homozygous and heterozygous samples, respectively, are significantly different. In fact, the HRM technique is so sensitive that, in most cases, it can even detect single base variations between homozygous samples.

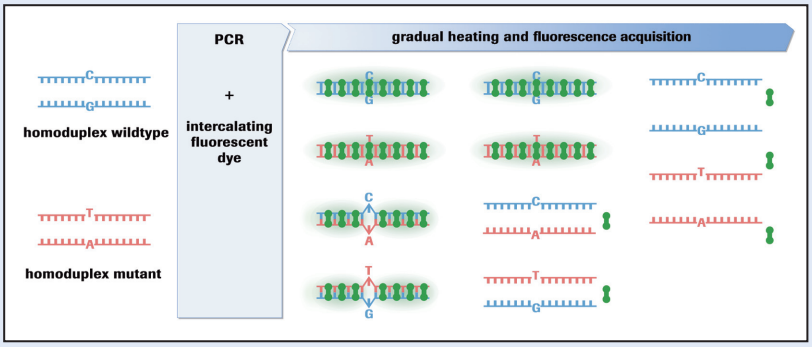


Figure 3: Because of a sequence mismatch, heteroduplex DNA melts at a different temperature than homoduplex DNA. This temperature difference can be detected in the presence of LightCycler® 480 ResoLight Dye, present in the LightCycler® 480 High Resolution Melting PCR Master Mix.

Setting up PCR for HRM-based Gene Scanning

Data derived from the High Resolution Melting curve must be analyzed sophistically to create meaningful results. This is true for all applications of High Resolution Melting, here just to mention gene scanning. Such analysis requires all experimental parameters to be rigorously controlled and highly reproducible from sample to sample. Thus, the first step in obtaining optimal results is choosing a real-time PCR instrument which can provide this reproducibility.

The LightCycler® 480 Instrument is an integrated, high-throughput real-time PCR platform fulfilling these requirements. Its innovative temperature control technology ensures sample-to-sample reproducibility, due to its ability to homogeneously heat the entire thermal block (Figure 4).

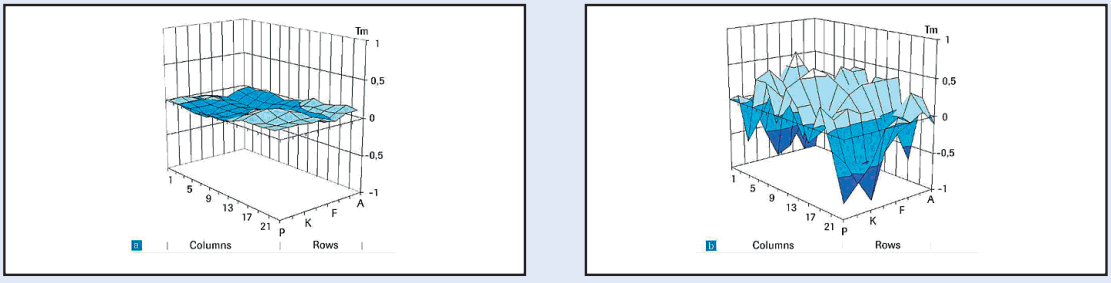


Figure 4: Comparison of temperature homogeneity over the multiwell plate on a LightCycler® 480 Instrument or an instrument from another supplier. Data were collected during the heating phase of an HRM analysis.

All HRM experiments have one thing in common: results are highly dependent on the quality of the individual PCR product. Thus, the second step in obtaining optimal HRM results is setting the PCR up properly.

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

1. **Carefully design the experiment**
2. **Choose the best primers**
3. **Standardize sample preparation**
4. **Optimize the reaction mixture**
5. **Optimize the PCR and melting programs**
6. **Analyze the experimental data with LightCycler® 480 Gene Scanning Software**
7. **Use additional tools and downstream techniques to get more detailed information**

1. Carefully design the experiment

Gene Scanning allows you to screen defined sections of DNA for any sequence variation or modification compared to other DNA samples. For an efficient screening assay, you must design your experiment carefully.

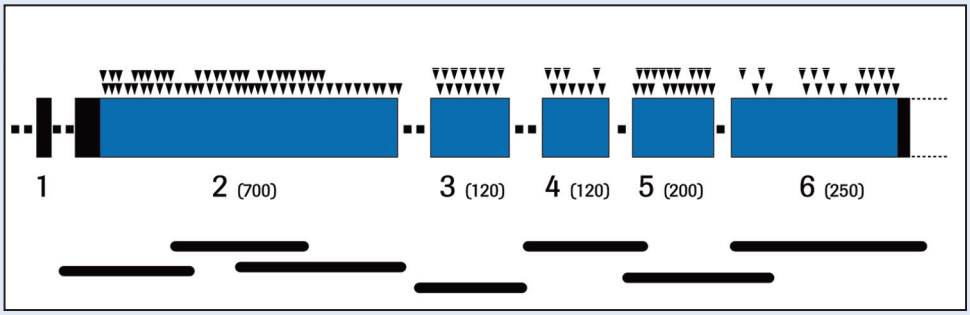


Figure 5: This typical gene, which has exons of differing lengths, requires careful placement of primers to get appropriate amplicons for the HRM analysis.

The following guidelines will help you design a gene scanning experiment to analyze a typical gene (*e.g.*, the one in Figure 5):

- If the exon to be scanned is not too long (maximum length 400 bp, ideally < 250 bp; *e.g.*, exons 3–6 in Figure 5), place the primers in the adjacent introns. This allows the entire exon to be scanned as a single amplicon.
- Longer exons (*e.g.*, exon 2, Figure 5) should be divided in 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.

Note: 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. Choose the best primers

To obtain the best HRM results, not all predeveloped and published primers are optimally suited. Sometimes design of new primers and the optimization according to the prerequisites of real-time PCR is recommended to generate better results faster. In general, 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 (http://frodo.wi.mit.edu/primer3/input.htm), or
  - LightCycler® Probe Design Software 2.0
- 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 unspecific products like *e.g.*, 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.

Formation of secondary structures in single-stranded or partially denatured DNA can have an influence on primer binding, amplification efficiency or subsequent melting.This may cause poor grouping or bad resolution.

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

3. Standardize sample preparation

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, *e.g.*, by doing the following:

- Use the same extraction procedure for all samples.
- Use nucleic acid preparation techniques that are highly reproducible. For example, you could use either:
  - the MagNA Pure LC Instrument or the MagNA Pure Compact Instrument 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 (Figure 6).

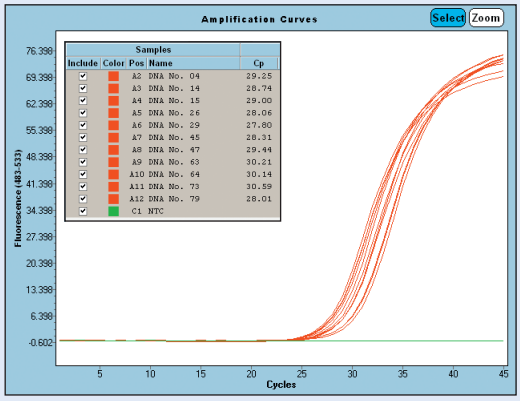


Figure 6: For good HRM analysis, all amplification curves should produce a crossing point of < 30. More importantly, ensure that all curves reach a similar plateau height.

4. Optimize the reaction mixture

Here are some general guidelines for optimizing the reaction mixture:

- To simplify optimization, we recommend to use 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.
- Salts affect DNA melting behavior, so it is important that the concentrations of buffer, Mg^{2+} 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 (Figure 7). A separate 25 mM $MgCl_2$ stock solution, supplied with the master mix, allows you to easily perform these titrations.
- You can verify the quality of the amplicons on an agarose gel or by standard melting curve analysis.

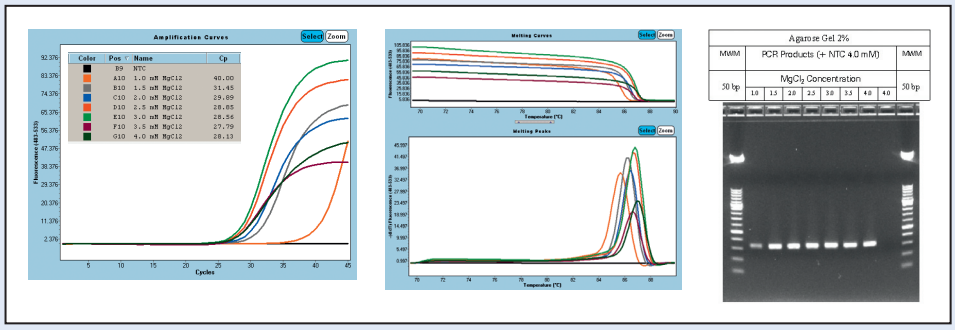


Figure 7: This example shows a typical Mg^{2+} titration. In this case, 3.0 mM (see green curve) would be the best choice because of low Cp value and high plateau height.

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. 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 °C and 55 °C (Figure 8).

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 makes sure that adequate amounts of PCR product are finally obtained.

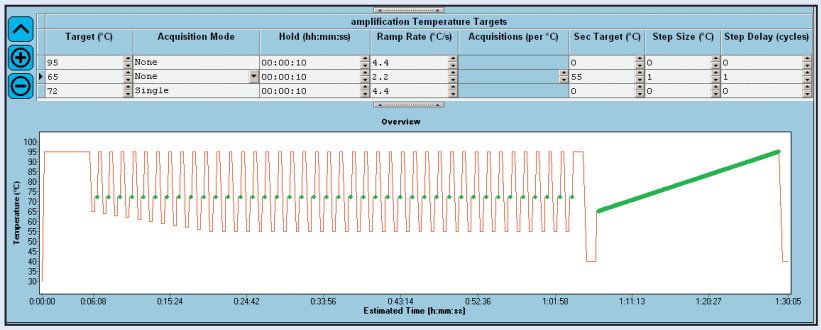


Figure 8: This is an example of a touchdown PCR program and profile.

Melting program:

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

- Rapidly cooling of 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 make sure that the melting program starts at least 10 °C before and ends at least 5 °C after the expected Tm value.
- Twenty-five acquisitions / °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 9: This example shows a typical High Resolution Melting protocol.

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 (Figure 10) by setting pre-melt (initial fluorescence) and post-melt (final fluorescence) signals of all samples to uniform values. In the next step (temperature shift, Figure 10), 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, Figure 10) involves subtracting the shifted, normalized curves from a reference curve (also called "base curve") to get 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.

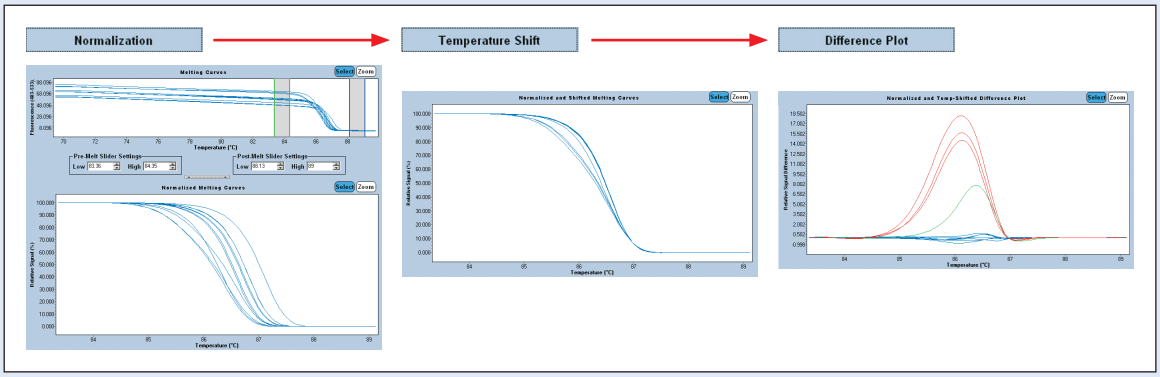


Figure 10: The Gene Scanning Analysis Software Module enables HRM analysis by 1) normalizing the raw melting curve data, 2) shifting the normalized curves along a temperature axis, and 3) plotting the melting curve differences between the various samples. In the resulting difference plot, samples can 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 unspecific by products created during amplification.

7. Advanced applications and downstream techniques

a. 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 very similarly shaped curves that are hard to distinguish.

The LightCycler® 480 Gene Scanning Software can easily distinguish heterozygous from homozygous samples. However, 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 to that problem, ensuring clear differentiation of homozygous variants (Figure 11).

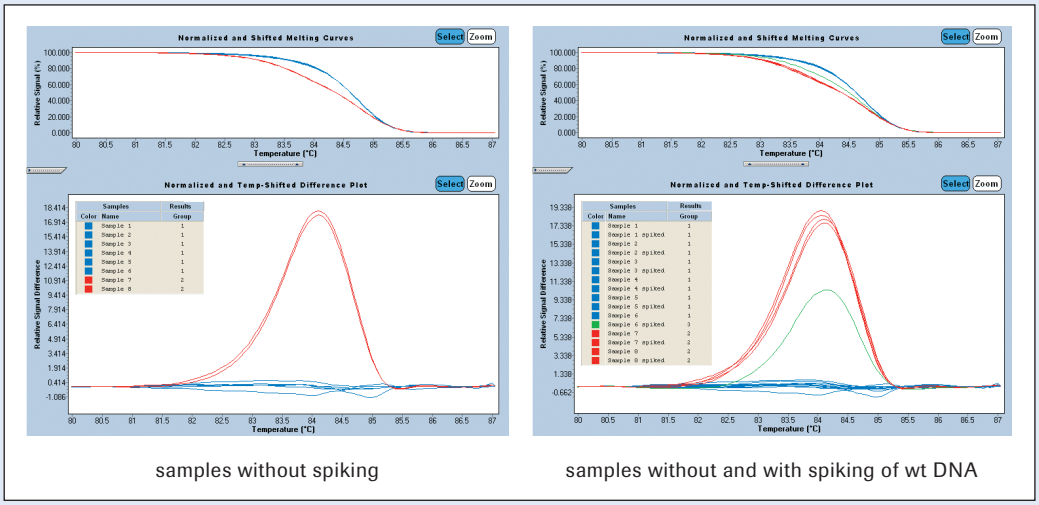


Figure 11: SNP analysis: Adding a fixed amount (between 10-20%) of a known homozygous DNA to all unknown samples prior to PCR enables to distinguish the melting curves of different homozygous genotypes and also the heterozygous. (blue: homozygous wildtype, red: heterozygous, green: homozygous mutant).

b. Refining results

If the automatic analysis algorithm does not resolve clustered samples, you can refine the gene scanning result by adjusting the sensitivity value (Figure 12). The sensitivity function influences the stringency with which melting curves are classified into different groups. In most cases, a high sensitivity value will produce more groups than a low value. The default sensitivity setting (0.30) generally strikes a reasonable balance between highly sensitive differentiation of all sequence variations and avoiding different groups with samples of the identical sequence.

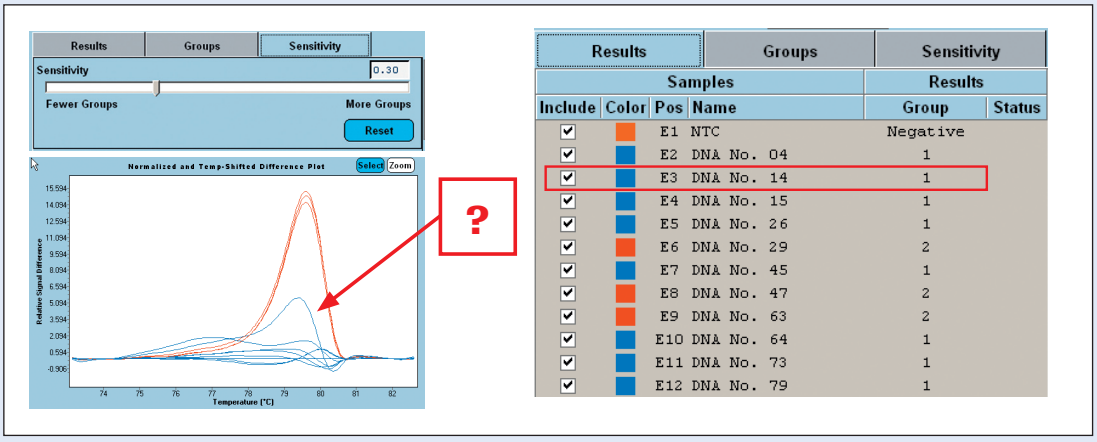


Figure 12a: The default sensitivity setting is 0.30. The sample indicated by an arrow gives an unclear result.

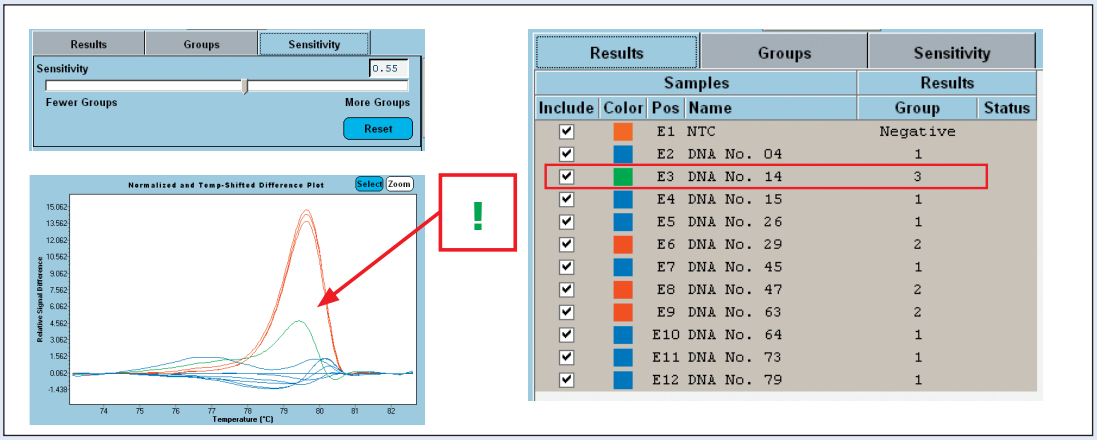


Figure 12b: Changing the sensitivity setting clarifies the situation. The sample of interest is now shown as sequence variant in the result list!

c. Using downstream applications (*e.g.*, 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. The LightCycler® 480 ResoLight Dye does not interfere with the sequencing process. 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).

Conclusions

Currently, High Resolution Melting is a rapidly developing research method. Consequently, we do not yet know how many HRM applications are adaptable to more routine procedures, amenable to automation, or capable of generating a result without manual interpretation/adjustment of the difference plots generated by HRM analysis software.

However, the simple guidelines presented here will allow you to optimize PCR for HRM. Following these guidelines will significantly reduce the amount of time required to obtain high-quality data and make the technique more suitable for high throughput applications. Last but not least, optimization of PCR is the key to this technology and sometimes even small changes as shifting a primer by one base can make a big difference in resolution.

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Notes

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