



# LightCycler<sup>®</sup> 480 Gene Scanning Software

Version 1.5



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# Prologue

## I Revision History

Version	Revision Date
1.0	February 2007
2.0	February 2008


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Questions or comments regarding the contents of this manual can be directed to the address below or to your Roche representative.

Roche Diagnostics GmbH  
Roche Applied Science  
Global Customer Support  
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82372 Penzberg, Germany

Every effort has been made to ensure that all the information contained in the *LightCycler® 480 Gene Scanning Software* manual is correct at the time of printing. However, Roche Diagnostics GmbH reserves the right to make any changes necessary without notice as part of ongoing product development.

## II Contact Addresses

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	Distribution in the US	Roche Diagnostics 9115 Hague Road PO Box 50457 Indianapolis, IN 46250 USA

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With the LightCycler® 480 Gene Scanning Software, the user can detect heteroduplex structures in DNA samples on the LightCycler® 480 Instrument. This assay requires only routine PCR amplification of the sample followed by a simple, rapid High Resolution Melting experiment in the presence of LightCycler® 480 High Resolution Melting Dye. After the experiment, the software analyzes the shape of the melting curves and groups those that are similar. Samples with known genotype can be included as melting standards in the experiment, allowing the user to determine genotypes of unknown samples in the analysis.

The LightCycler® 480 Gene Scanning Software is intended for general laboratory use in combination with the LightCycler® 480 Instrument and LightCycler® 480 Software.

### V License Statements for the LightCycler® 480 Gene Scanning Software

Parts of the Software used for the LightCycler 480 System are licensed from Idaho Technology Inc., Salt Lake City, UT, USA.

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Read the following terms and conditions of this Software License Agreement (“Agreement”) carefully before installing the LightCycler® 480 Software, hereinafter referred to as (“Software”). Proceeding with the installation of the Software will constitute acceptance of the terms and conditions of this Agreement. By accepting the terms and conditions of this Agreement, the end-user (“Licensee”) assumes all responsibility and liability for the selection of this Software to achieve the intended results, and for its installation and subsequent use. If Licensee is not willing to be bound by the terms and conditions of this Agreement, the Software package must be promptly returned to Roche (“Supplier”) with a copy of the receipt against refunding of the purchase price for this Software.

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The headings of the several Sections of this Agreement are intended for convenience of reference only and are not intended to be a part of or to affect the meaning or interpretation of this Agreement.

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This Agreement shall be governed by and construed in accordance with the laws of the State of Indiana, without giving effect to any choice of law principles thereof. The parties agree that the United Nations Convention on Contracts for the International Sale of Goods (1980) is specifically excluded from application to this Agreement.

## VII Conventions Used in this Manual





### Text Conventions

To impart information that is consistent and memorable, the following text conventions are used in this Operator's Manual:

Numbered listing	Steps in a procedure that you must perform in the order listed.
Italic type, blue	Points to a different chapter in this Operator's Manual, which you should consult.
Italic type	Points to a software function or element.

### Symbols

In this Manual, these symbols are used to indicate information that deserves your special attention.

Symbol	Heading	Description
	IMPORTANT NOTE	Information critical to the success of the procedure or use of the product.
	INFORMATION NOTE	Additional information about the current topic or procedure.
		Procedure continued on next page.
		End of procedure.

## VIII Warnings and Precautions

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The LightCycler<sup>®</sup> 480 System is equipped with software, enabling the user of the Product to connect it with a network. Roche draws the attention of the user to the fact that such connection may have an adverse effect on the Product's integrity, *e.g.*, due to an infection of the Product with malicious code (viruses, Trojan horses, etc.) or access by unauthorized third parties (*e.g.*, intrusion by attackers). Roche therefore highly recommends to protect the Product against such risks by taking appropriate and state-of-the-art action. As the Product is not intended to be used within networks without an appropriate firewall and has not been designed for such use, Roche assumes no liability in that regard.

Roche offers the user the cobas IT firewall to be installed prior to the first connection of the Product to any network. For further information on this cobas IT firewall and/or the Roche network security concept please contact your local Roche representative.

In the event the user connects the Product with any network without using the cobas IT Firewall, Roche cannot offer any Product support regarding any problem resulting from such network connection. In case of a stand-alone use of the software of the Product on or in connection with other IT components (*e.g.*, installation on other PCs) Roche assumes no liability with respect to any interference of the user's networks and/or other IT components such use might have. Roche's liability for the proper functioning of the software under the respective license and/or purchase agreements with the user shall remain unaffected.



*Contact your local Roche representative for detailed information on the cobas IT firewall.*

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Tests indicate that Microsoft Office and Norton Antivirus software do not interfere with LightCycler<sup>®</sup> 480 Software and LightCycler<sup>®</sup> 480 software modules; these programs may be installed on the LightCycler<sup>®</sup> 480 Instrument PC. Do not install any other software on the LightCycler<sup>®</sup> 480 Instrument PC. Installation of any additional software on the LightCycler<sup>®</sup> 480 Instrument PC may interfere with the operation of the LightCycler<sup>®</sup> 480 Software and LightCycler<sup>®</sup> 480 software modules, and could affect data security.

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**Anti-virus software is not provided.** Therefore, it is essential to take precautions to ensure that any software loaded onto the system is virus free.

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## A Gene Scanning with the LightCycler® 480 System

“Gene scanning” or “mutation scanning” techniques detect the presence of sequence variation in target-gene derived PCR amplicons. “Gene Scanning” is based on “High Resolution Melting”, a novel, closed-tube post-PCR (Polymerase Chain Reaction) method enabling genomic researchers to analyze genetic variations in PCR amplicons prior to or as an alternative to sequencing. High Resolution Melting provides high specificity, sensitivity and convenience at significantly higher speed and much lower cost than other established (e.g., gel-based) methods. For example, in a diploid genome, equivalent regions from maternal and paternal chromosomes are both amplified by the Polymerase Chain Reaction. The PCR products can then be analyzed for completely matched hybrids (called homoduplexes) and mismatched hybrids (heteroduplexes).

In a LightCycler® 480 System Gene Scanning experiment, sample DNA is first amplified via real-time PCR in the presence of LightCycler® 480 High Resolution Melting Dye. Immediately after DNA amplification, a High Resolution Melting experiment can be performed on the same LightCycler® 480 Instrument and analyzed with LightCycler® 480 Gene Scanning Software to identify sequence variants. Thus, the entire mutation screening process is homogeneous. That is, the entire experiment can be done on the LightCycler® 480 Instrument; post-PCR analysis does not require a separate device.

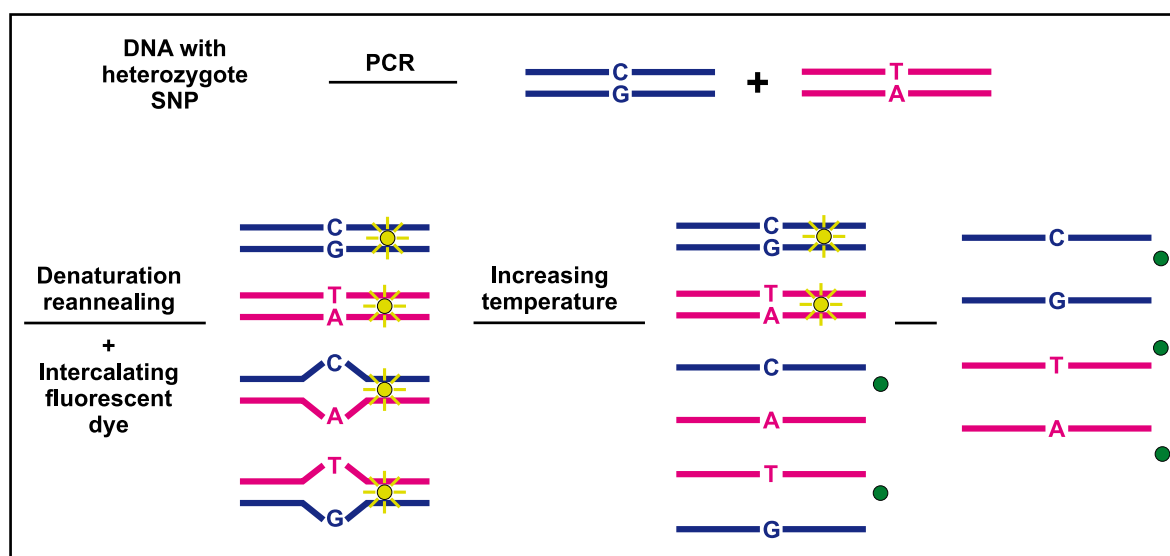
# 1 High Resolution Melting

The key technique in gene scanning, High Resolution Melting, is a refinement of earlier, well-established DNA dissociation (or “melting”) techniques (*e.g.*, to determine the  $T_m$  of a DNA hybrid). Like all melting analyses, the technique subjects DNA samples to increasing temperatures and records the details of their dissociation from double-stranded (dsDNA) to single-stranded form (ssDNA).

Before a High Resolution Melting analysis can be performed, the target sequence must be available in high copy number. The easiest way to accomplish this is to perform a DNA amplification reaction (PCR) before the High Resolution Melt.

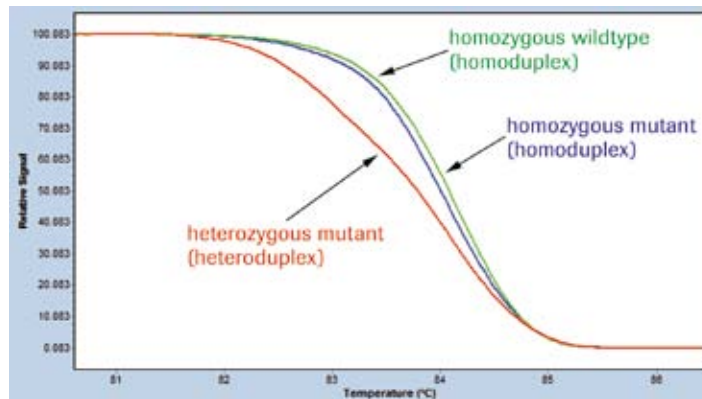
Both procedures are performed in the presence of a fluorescent dye that binds only dsDNA. The dye does not interact with ssDNA, but fluoresces strongly in the presence of dsDNA. This change in fluorescence can be used both to measure the increase in DNA concentration during PCR and then to directly measure thermally-induced DNA dissociation during High Resolution Melting.

For detection of sequence variations, differences in the melting curves of the amplicons are analyzed. Heterozygote DNA forms heteroduplexes that begin to separate into single strands at a lower temperature and with a different curve shape than homozygote DNA. Depending on the individual sequence, most of the different homozygotes give distinguishable melting curves, too.



In a melting experiment, fluorescence is initially high because the sample starts as dsDNA, but fluorescence diminishes as the temperature is raised and DNA dissociates into single strands. The observed “melting” behavior is characteristic of a particular DNA sample.

Mutations in PCR products are detectable because they change the shape of the melting curve. When the mutant sample is compared to a reference “wild type” sample, these changes are visible. Below is an example of an experiment with the LightCycler® 480 Instrument and the LightCycler® 480 High Resolution Melting Dye that identifies a single nucleotide polymorphism (SNP).



This figure shows how a High Resolution Melting experiment can detect both homozygous and heterozygous allelic variants in a sample. Homozygous variants are detectable because their melting curves are displaced along the temperature axis (x-axis) relative to homozygous “wild type” samples. Heterozygous variants have melting curves that differ even more dramatically in shape from “wild type” curves.



*In heterozygous samples, melting curve shape changes because the observed melting curve is actually a composite of both heteroduplex and homoduplex components. Heteroduplexes formed in the sample (i.e., between the “wild type” and variant strands) are less stable than the homoduplexes formed, and thus, dissociate more readily.*



## 2 Components of the LightCycler® 480 System Gene Scanning Assay

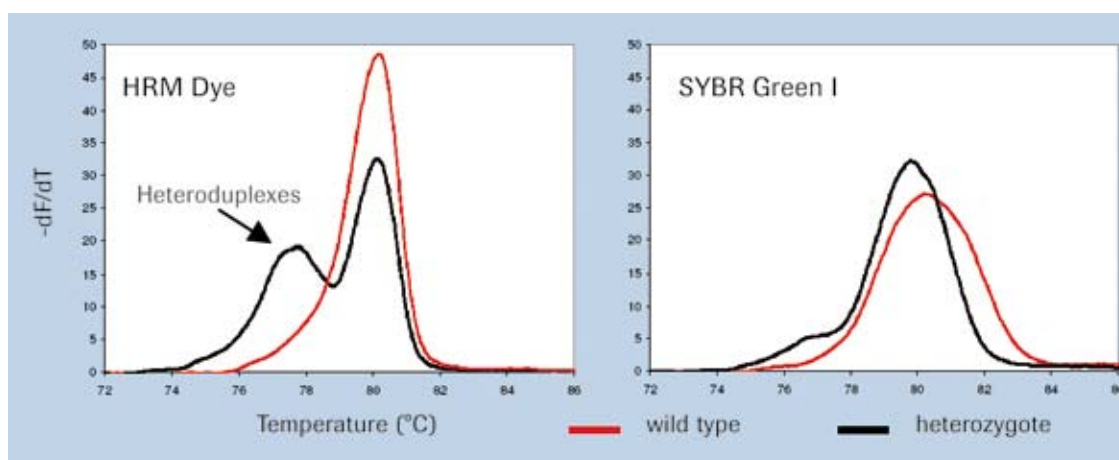
To obtain meaningful gene scanning results from High Resolution Melting analysis, three components (the DNA-binding dye, the analytical software, and the real-time PCR instrument itself) of the LightCycler® 480 System must work optimally. Here is a brief look at how these three components have been engineered to work together well in a LightCycler® 480 System Gene Scanning experiment.

### 2.1 LightCycler® 480 High Resolution Melting Dye

High Resolution Melting analysis relies on a new generation of dsDNA binding dyes. LightCycler® 480 High Resolution Melting Dye is a member of this new dye family. This unique dye can detect the presence of heteroduplexes formed during PCR (e.g., if the sample is heterozygous for a particular mutation). This feature is not shared with other dyes traditionally used in real-time PCR (e.g., SYBR Green I or ethidium bromide).

LightCycler® 480 High Resolution Melting Dye is not toxic to amplification enzymes. Thus, high concentrations of the dye do not affect the PCR. These high concentrations completely saturate the dsDNA in the sample.

dsDNA remains dye-saturated during the subsequent melting experiment. Under these conditions, even small changes in the melting behavior result in subtle, but reproducible changes in High Resolution Melting Dye fluorescence.



According to Wittwer *et al* (2003)<sup>1</sup>, this occurs because the dye cannot redistribute itself from denatured to non-denatured regions of the DNA during melting. Further, the dyes no longer show a preference for products that melt at higher temperatures.

High fidelity correlation between fluorescence changes and DNA melting increases the resolution of the recorded melt profiles.

<sup>1</sup> Carl T. Wittwer, Gudrun H. Reed, Cameron N. Gundry, Joshua G. Vandersteen, and Robert J. Pryor (2003). High-Resolution Genotyping by Amplicon Melting Analysis Using LCGreen. *Clinical Chemistry* 49, 853–860

## 2.2 Data Analysis using the LightCycler® 480 Gene Scanning Software

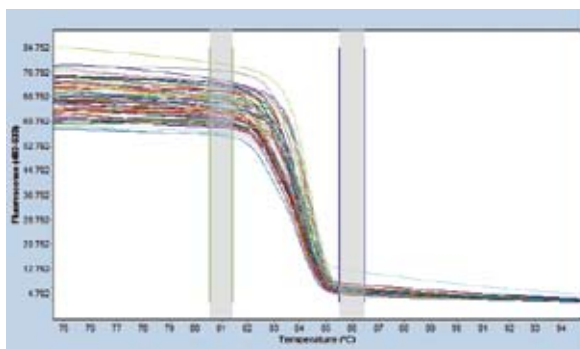
LightCycler® 480 Gene Scanning Software analyzes the High Resolution Melting curve data to identify changes in the shape of the curve, which indicate the presence of sequence variations in the PCR product.



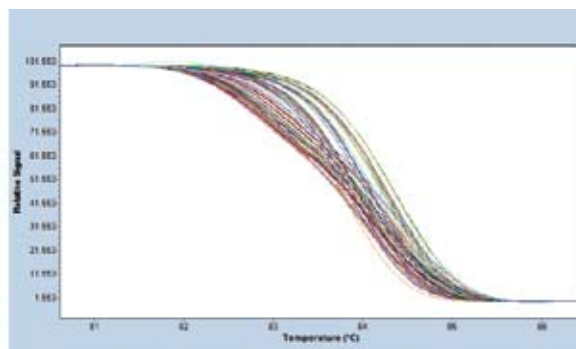
*Correct interpretation of the data depends to a large extent on the software algorithms used. LightCycler® 480 Gene Scanning Software has been developed specifically to provide the most accurate analysis of High Resolution Melting curves.*

The standard workflow followed by the LightCycler® 480 Gene Scanning Software has four basic steps:

1. **Detecting negatives:** LightCycler® 480 Gene Scanning Software automatically uses a negative filter to detect negative samples, *i.e.*, samples with low fluorescence signals that lack a prominent melting curve. The software also allows you, if you wish, to identify the negative samples in the run manually.
2. **Normalizing:** The second step in the analysis is to normalize the raw melting curve data by setting the pre-melt (initial fluorescence) and post-melt (final fluorescence) signals of all samples to uniform values. Pre-melt signals are uniformly set to a relative value of 100%, while post-melt signals are set to a relative value of 0%. Normalizing the initial and final fluorescence in all samples aids interpretation and analysis of the data. In some cases, samples with homozygous SNPs may be distinguished from the wild type by the displacement of their melting curves, which is easier to see in the normalized data.

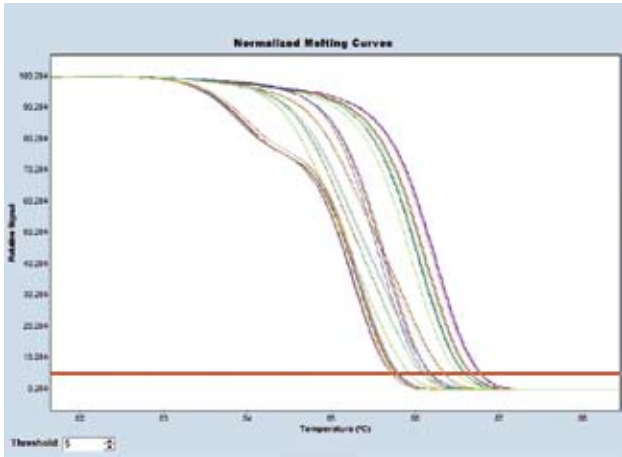


**unnormalized melting curves**

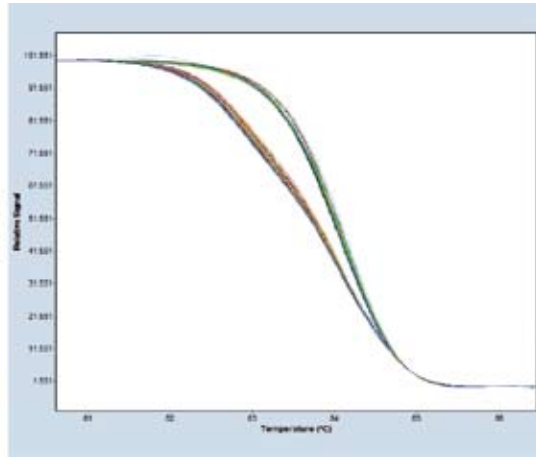


**normalized melting curves**

3. **Temperature shifting:** The next step is to shift the temperature axis of the normalized melting curves at the point where the entire double-stranded DNA is completely denatured. For this, the software automatically applies a default Temp Shift Threshold of 5% to all data. (If you wish, you can set this threshold manually to a different value.) Now, samples with heterozygous SNPs can easily be distinguished from the wild type by the different shapes of their melting curves.

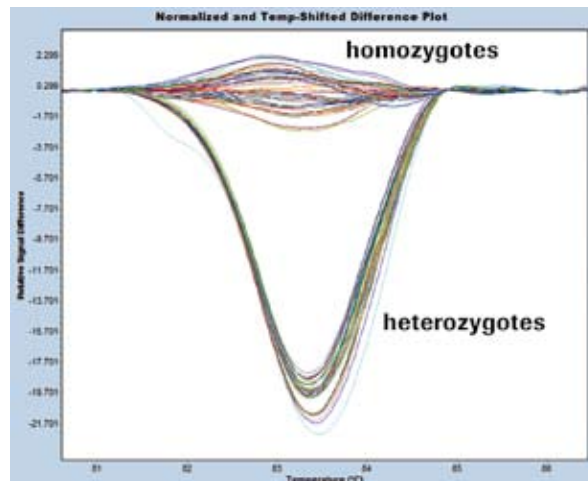


normalized melting curves



normalized, temp-shifted melting curves

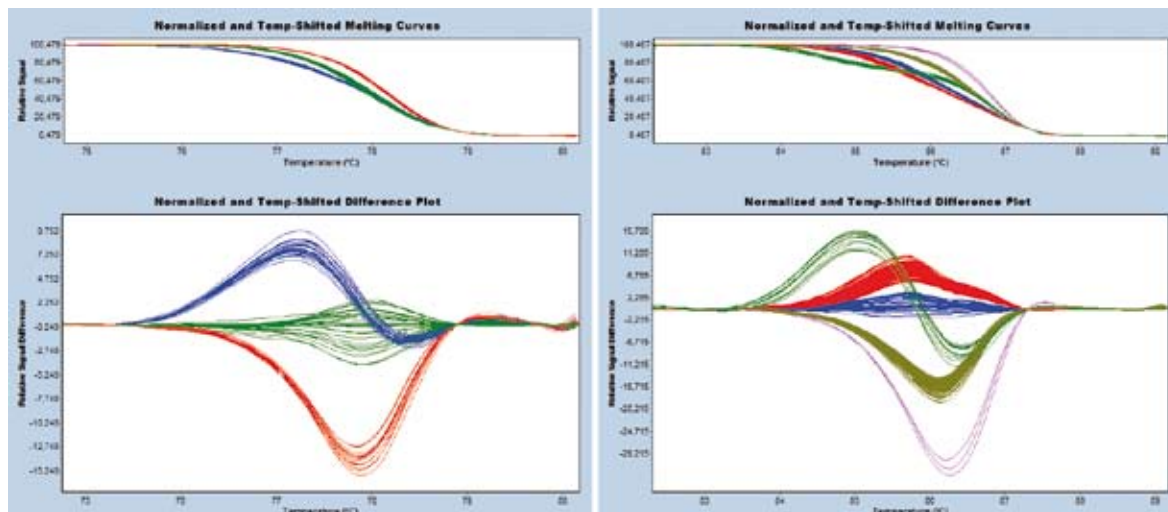
4. **Difference Plot:** The final step is to further analyze the differences in melting curve shape by subtracting the curves from a reference curve (also called “base curve”), thus generating a Difference Plot, which helps cluster samples automatically into groups that have similar melting curves (e.g., those with the same genotype).



The way melting curves for homozygotes and heterozygotes are plotted depends on the base curve you selected for the Difference Plot. In the example above, a homozygote sample was selected as base curve, resulting in negative melting curves for the heterozygotes. (This is because heterozygotes melt at a lower temperature than homozygotes.) If you selected a heterozygote sample as base curve, homozygotes would appear as positive melting curves in the example shown.

The following examples demonstrate gene scanning:

- ▶ For the single-nucleotide polymorphism (SNP) G→T in the LPLH3 gene (163 bp amplicon), resulting in 3 main variant groups.
- ▶ For sequence variations in the MBL2 gene (219 bp amplicon), resulting in 4 main variant groups corresponding to the 4 most frequent haplotypes described for this gene in literature and 3 samples of a further genetic variant.



### 2.3 LightCycler® 480 Instrument

High Resolution Melting analysis of nucleic acid requires that the analyzing instrument is able to detect even small changes in fluorescence as a function of temperature-induced DNA melting. In the LightCycler® 480 Instrument, images of DNA melting (*i.e.*, fluorescence) are captured by a sensitive CCD camera and magnified to reveal subtle details in DNA melting profiles. These profiles are then compared from sample to sample to determine relationships between the samples.

The optical components of the LightCycler® 480 System work together with its accurate thermal control system to generate highly reproducible results from sample to sample. Since the samples are held in a multiwell plate, the LightCycler® 480 System can analyze many samples in a single run.

### 3 Advantages of the High Resolution Melting Technique

Melting curve analysis is based on a robust, post-PCR physical measurement and, therefore, offers several advantages over mutation detection methods that derive information from the amplification process itself:

- ▶ Any amplicon can be screened for unknown sequence variants with a single high-resolution dye; you do not need to target a specific variant with allele-specific probes.
- ▶ You can design a genotyping assay on the basis of less sequence data.
- ▶ Each reaction generally reveals more information.

In addition, the homogeneous High Resolution Melting technique can process more samples more conveniently than traditional, non-homogeneous methods (*e.g.*, dHPLC or SSCP) which require amplicons to be screened for sequence variants on a separate instrument after PCR.

High Resolution Melting analysis identifies heterozygous single-base changes in PCR products with a sensitivity and specificity that is comparable or superior to non-homogeneous techniques. Variants can be detected regardless of their position within the fragment.

Identification of homozygous sequence alterations is more difficult; most other mutation scanning methods are unable to distinguish these alterations from wild type sequences. By contrast, High Resolution Melting has identified homozygous sequence alterations in several different types of amplicons.

## B Preparing a LightCycler® 480 System Gene Scanning Experiment

### 1 Designing the Gene Scanning Assay

These guidelines will help you design an effective Gene Scanning assay:

- ▶ A single base variation affects the melting behavior of a 100 bp amplicon more than a 500 bp amplicon; thus, short amplicons are more likely to show the effects of small sequence changes. Therefore, it is recommended to select PCR primers that amplify a relatively short sequence (100 - 250 bp).



*Primers should anneal at temperatures around 60°C.*

Nevertheless, it is possible to target longer sequences (up to 500 bp), but keep in mind that analysis of these products will usually have lower resolution. In addition, such products (>250 bp) are more likely to contain multiple melting domains and generate complicated melting curves.

- ▶ Secondary structures can affect the efficiency of the amplification reaction. Therefore, it might be of advantage to determine the folding characteristics of both primers and amplicon with software that can profile secondary structures.



*Make sure to set the folding temperature equal to the annealing temperature that will be used for the reaction (e.g., 60°C).*

The *DINAMelt Server* from *Rensselaer Polytechnic Institute* provides appropriate software for such secondary structure analyses; this software can make corrections for both salt and magnesium concentration (see <http://www.bioinfo.rpi.edu/applications/hybrid/twostate.php>).

Low delta-G values indicate a high level of secondary structure. Strands with high delta-G values produce less secondary structures and so are favored in the amplification reaction. For best results, the delta-G values should be above -1.

## 2 Sample Material

Because a LightCycler® 480 System Gene Scanning experiment involves comparing melting profiles from independent PCR reactions, it is crucial to minimize reaction-to-reaction variability. Standardizing the template DNA is one means of minimizing variability. Follow these guidelines when preparing or handling template DNA:

- ▶ Use isolation and storage procedures that minimize the potential for sample degradation. Avoid procedures that can introduce excessive amounts of inhibitors (*e.g.*, due to ethanol carry-over).
- ▶ If extraction is required, use the same extraction procedure to prepare all samples to be analyzed via High Resolution Melting. This eliminates any subtle differences that might be introduced by the reagent components in the final elution buffers of different extraction procedures.



*For reproducible isolation of nucleic acids 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.*

*For details see the Roche Applied Science Biochemicals catalog or home page, <http://www.roche-applied-science.com>.*

- ▶ Resuspend all DNA samples in the same buffer, quantify them using spectrophotometry, and adjust them to the same concentration with the resuspension buffer. Salts affect DNA melting behavior, so it is important that the concentrations of buffer, Mg<sup>2+</sup> and other salts in the reaction mix are as uniform as possible for all samples.
- ▶ Use the same amount of template in each reaction. The recommended amount is 5 to 30 nanograms of template DNA in a 20 µl reaction volume, which should produce amplification plots with a Cp value of no more than 30 cycles. Products that reach this threshold at higher Cps (due to insufficient amounts of starting template or template degradation) typically produce variable High Resolution Melting results due to amplification artifacts.
- ▶ If you are using archival genomic DNA, repurify the DNA by binding it to silica (*e.g.*, with the High Pure PCR Template Preparation Kit from Roche Applied Science) and eluting it into a fresh buffer before using it in a Gene Scanning experiment. This will eliminate artifacts caused by sublimation (the direct transition of frozen material to gas), which frequently occurs in such samples and concentrates salts and other material that affect both amplification and High Resolution Melting.

### 3 PCR Primers

- ▶ Design PCR primers that have annealing temperatures around 60°C and produce short amplicons (100–250 bp). Use a software package like *Primer3* (see <http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 WWW.cgi>) or *LightCycler® Probe Design Software 2.0* for designing the primers.

Use primers that have been purified by HPLC.

- ▶ To avoid primer-dimer formation, use relatively low primer concentrations (less than 300 nM) in the experimental reactions.
- ▶ Depending on the amount of specific product observed in the initial experiment, try repeating the experiment with a series of primer dilutions, increasing or decreasing the concentration in 0.1 µM steps. If initial production of the specific product is robust, you might try lower concentrations of the primers. If initial production of specific product is weak, try higher concentrations of the primers.
- ▶ You do not need to use primers with GC clamps; they will not improve High Resolution Melting.
- ▶ BLAST the primer sequences to ensure they are specific for the target species and gene (see <http://www.ncbi.nlm.nih.gov/BLAST/>).



## 4 PCR Reagents

Hot-start PCR techniques are strongly recommended for High Resolution Melting applications, since they avoid the formation of non-specific amplification products at the beginning of the reaction.

Roche Applied Science provides a convenient, 2× concentrated master mix for such hot start procedures. This LightCycler® 480 High Resolution Master contains FastStart Taq DNA Polymerase and the High Resolution Melting Dye in a reaction buffer that contains no MgCl<sub>2</sub>. The Master is compatible with additives (e.g., DMSO) that enhance amplification of GC-rich sequences.

FastStart Taq DNA Polymerase is a chemically modified form of thermostable recombinant Taq DNA polymerase that shows no activity at temperatures up to 75°C. The enzyme is active only at high temperatures, where primers do not bind non-specifically. The enzyme is completely activated (by removal of blocking groups) during a single pre-incubation step (95°C, 5 minutes) before cycling begins.

A separate 25 mM MgCl<sub>2</sub> stock solution, supplied with the Master, allows you to easily optimize the Mg<sup>2+</sup> concentration. Determining the optimal MgCl<sub>2</sub> concentration is essential to ensure both the specificity and robustness of the PCR. The optimum MgCl<sub>2</sub> concentration for a LightCycler® 480 High Resolution Melting assay may vary from 1.5 to 3.5 mM. Therefore, we strongly recommend that you titrate the MgCl<sub>2</sub> concentration in the reaction between 1.5 and 3.5 mM (in 0.5 mM steps) when establishing a new assay. Determine the specificity of each PCR by agarose gel electrophoresis.



*The amount of MgCl<sub>2</sub> in a reaction will affect the shape and  $T_m$  of the melting profile. In some cases, fragments that melt with a single inflection at low MgCl<sub>2</sub> concentrations may show multiple inflections at high MgCl<sub>2</sub>. While we observed this infrequently, you should be aware of this possibility. As a general rule, at higher MgCl<sub>2</sub> concentrations, a fragment will melt with a slightly higher overall  $T_m$ .*

## 5 PCR Parameters

Data obtained from High Resolution Melting is only as good as the amplification product being analyzed. Nothing is more critical to High Resolution Melting data than having robust amplification of a single product. The High Resolution Melting Dye binds all double-stranded DNA present in a reaction. Specific amplification product, undesired amplification product(s), and primer-dimer products all bind the dye with equal affinity; thus all contribute to the overall fluorescence and melting profile.

To optimize the amplification, determine the best thermal cycling parameters. If you use the LightCycler® 480 High Resolution Master to establish your LightCycler® 480 Gene Scanning assay, you can use the following parameters in your initial experiments:

Setup				
Detection Format	High Resolution Melting Dye			
Programs				
Program Name	Cycles		Analysis Mode	
Pre-Denaturation	1		None	
Amplification	45 <sup>1)</sup>		Quantification	
High Resolution Melting	1		Melting Curves <sup>6)</sup>	
Cooling	1		None	
Temperature Targets				
Target [°C]	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s) (96-well / 384-well)	Acquisitions (per °C)
Pre-Incubation				
95	None	00:10:00	4.4 / 4.8	-
Amplification				
95	None	00:00:10	4.4 / 4.8	-
primer dependent <sup>2)</sup>	None	00:00:10	2.2 / 2.5	-
72	Single	00:00:10 - 00:00:20 <sup>3)</sup>	4.4 / 4.8	
High Resolution Melting				
95	None	00:01:00	4.4 / 4.8	
40 <sup>4)</sup>	None	00:01:00	2.2 / 2.5	
60 <sup>5)</sup>	None	00:00:01	1	-
95 <sup>5)</sup>	Continuous	-	-	25
Cooling				
40	None	00:00:10	4.4 / 4.8	-

In case you do not know the melting temperatures of your PCR primers exactly, it is recommended to apply a touchdown PCR protocol covering a range of annealing temperature from 65 to 53°C. Modify the *Temperature Targets* of the *Amplification* program as shown in the table below:

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s) (96-well / 384-well)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
95	None	00:00:10	4.4 / 4.8	-	0	0	0
65	None	00:00:10	2.2 / 2.5	-	53	0.5	1
72 <sup>3)</sup>	Single	00:00:10 - 00:00:20	4.4 / 4.8	-	0	0	0

#### 1) Number of cycles

45 cycles are suitable for most assays. If the assay is optimized and has steep amplification curves and early crossing points (even when target concentrations are low), 40 cycles should be sufficient. Reducing the number of cycles will reduce the time required for the assay.

#### 2) Annealing temperature

Annealing temperature is the parameter that most influences specificity and robustness of amplification. For initial experiments set the target temperature (*i.e.*, the primer annealing temperature) 2°C below the calculated primer  $T_m$ . The amount of specific product, the presence/absence of undesirable side product, and the presence/absence of dimer product in these experiments will dictate the best way to optimize this parameter. If the reaction produces undesirable product, increase the annealing temperature. If amplification is not robust, decrease the annealing temperature and/or increase the duration of the annealing step.

#### 3) Elongation time

Calculate the exact elongation time required for your specific target by dividing the amplicon length by 25 (*e.g.*, a 500 bp amplicon requires 20 s elongation time).

#### 4) Melting pre-hold step

This pre-hold temperature ensures that all PCR products have re-associated and encourages heteroduplex formation.

#### 5) Melting interval

Actual melting conditions depend upon the amplicon. For initial experiments set a wide melting interval, *e.g.*, from 60 to 95°C. Once you have determined where the product will melt, reduce the melting interval to approximately 25°C. Ensure that the melt program starts at least 10°C before and ends at least 10°C after the expected  $T_m$  value.

#### 6) Analysis mode

No special analysis mode for Gene Scanning assays is available. Gene Scanning experiments are performed in standard Melting Curves analysis mode.

## 6 Rapid Detection of Amplification Artifacts

The goal of a LightCycler® 480 System Gene Scanning assay is to generate a single pure PCR product. Any amplification artifacts (e.g., primer-dimers) may lead to misleading results.

On the LightCycler® 480 System, you can easily determine whether the PCR products include primer-dimers by comparing your samples to a non-template control. After the initial melting curve run, simply view the Melting Peaks plot (negative first derivative of the sample fluorescence plotted against temperature; generated by the  $T_m$  Calling Analysis Module of the LightCycler® 480 Software). That plot should show no peaks for the no-template control. If initial experiments show reaction product in your no-template control, reoptimize the reaction (e.g., by designing new primers) and repeat the experiment. For further verification, you may also want to examine the product on an electrophoretic gel.



*You can access the Melting Peaks plot directly from the LightCycler® 480 Gene Scanning Software module. There is no need to run a separate  $T_m$  Calling Analysis.*

## C Installing the LightCycler® 480 Gene Scanning Software

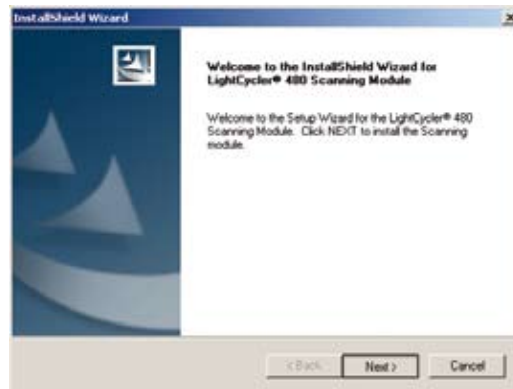
The LightCycler® 480 System Gene Scanning Software is provided as an additional module on a separate software CD. Software installation is performed using a self-extracting installation program. To install the software on a LightCycler® 480 control unit or on a non-Roche PC follow the steps below.



To enable installation and running of the LightCycler® 480 Gene Scanning Software, the LightCycler® 480 Software must be installed on the PC.

### To install the LightCycler® 480 Gene Scanning Software:

- 1 Make sure that you have the administration rights to install the software. Insert the LightCycler® 480 Gene Scanning Software CD. If installation does not start automatically, double-click *ScanningModuleInstall.exe*. The installation process transfers files, extracts the files, and prepares the installation wizard. The *InstallShield Wizard* window opens.

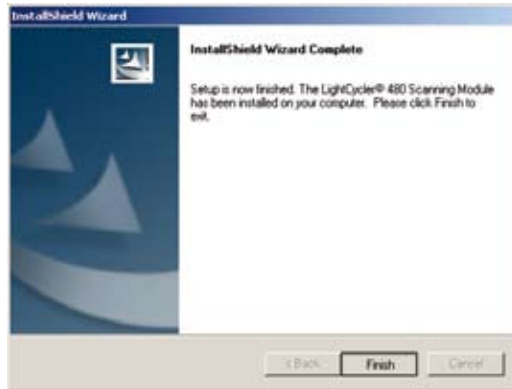


Click *Next*.

- 2 You are prompted to agree to the license conditions. Click *Yes*.



- 3 The LightCycler® 480 Gene Scanning Software is installed. When the installation process has finished, the *InstallShield Wizard Complete* window is displayed. Click *Finish*.



## D Performing Gene Scanning Analysis

You can perform a Gene Scanning analysis on any experiment that contains a melting curve program. The LightCycler® 480 Gene Scanning Software determines the heteroduplex structures in samples by analyzing experimental data generated in the presence of the LightCycler® 480 High Resolution Melting Dye. After samples are amplified by PCR and subjected to a melting curve experiment, the software analyzes the shapes of the individual curves and groups samples that have similar melting curves.

You can also include melting standards of known sequence variants or genotypes in your experiment. In this case, the software compares the melting curves of the individual samples to the designated in-run melting standards.



*Without the LightCycler® 480 Gene Scanning Software you can still view any gene scanning experiment in the LightCycler® 480 Software but you cannot perform a Gene Scanning analysis.*

### To perform a gene scanning experiment:

- 1 Set up an experiment containing an amplification program (to amplify the target samples) and a melting curve program (to melt the samples). Whether you need to include an internal standard in your experiment depends on the analysis mode you want to use:
  - ▶ Auto Grouping or Common/Variants analysis: no melting standards are required
  - ▶ In-run melting standards: include melting standard samples

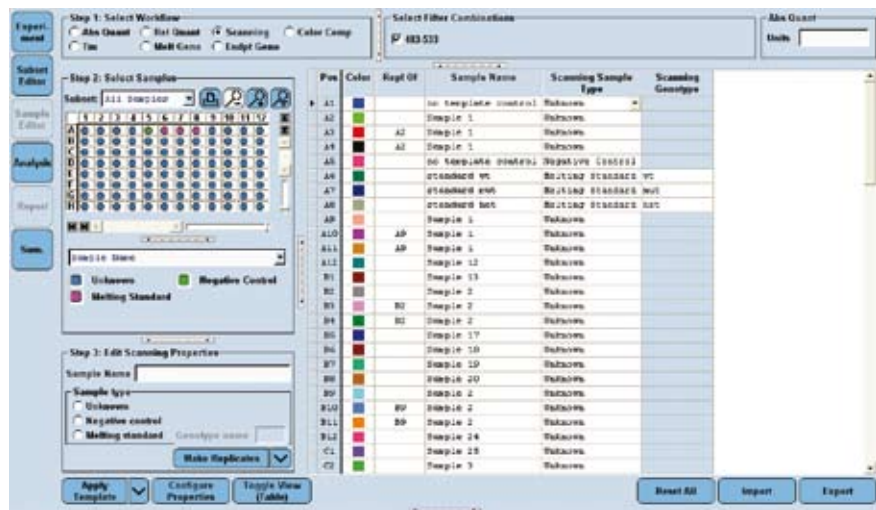
If you wish, you may also include control samples (e.g., negative controls or no-template controls) in your experiment.





*Internal standards act as positive controls.*



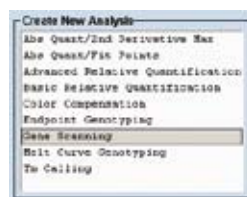
- Click *Sample Editor* in the *Module* bar, then select the workflow *Scanning*. After the workflow is selected, the LightCycler 480 Software automatically reconfigures the *Sample Editor*.



- Define the properties of the samples.  
For detailed information on the *Sample Editor* see the LightCycler® 480 Instrument Operator’s Manual, section “Entering Sample Information”.  
The software uses the following parameters for calculation:

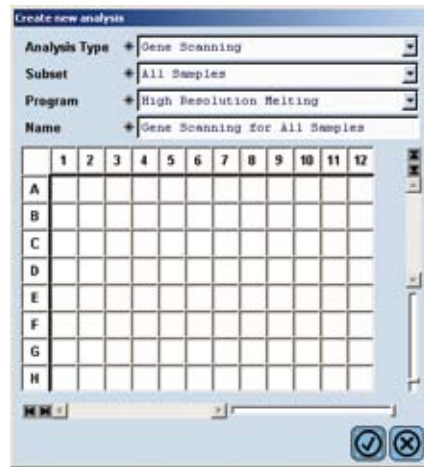
Column Name	Description	Valid Values
<b>Target Name</b>	Name of the (PCR) target (e.g., the name of the gene amplified and detected)	Alphanumeric value (≤ 25 characters); default value is “Target xxx-yyy”, where xxx and yyy are the excitation and emission wavelengths
<b>Sample Type</b>	Type of sample  <i>If you want to use the “In-run Standards” grouping option, you need to include a Melting Standard for any genotype you want to detect.</i>	Choose from dropdown list: ▶ Unknown ▶ Negative Control ▶ Melting Standard
<b>Genotype</b>	Genotype of melting standard or positive control samples  <i>This field is active only when the Sample Type is “Melting Standard”.</i>	Alphanumeric value (≤ 25 characters)

- Click *Analysis* in the *Module* bar. In the *Create New Analysis* list, select *Gene Scanning*.

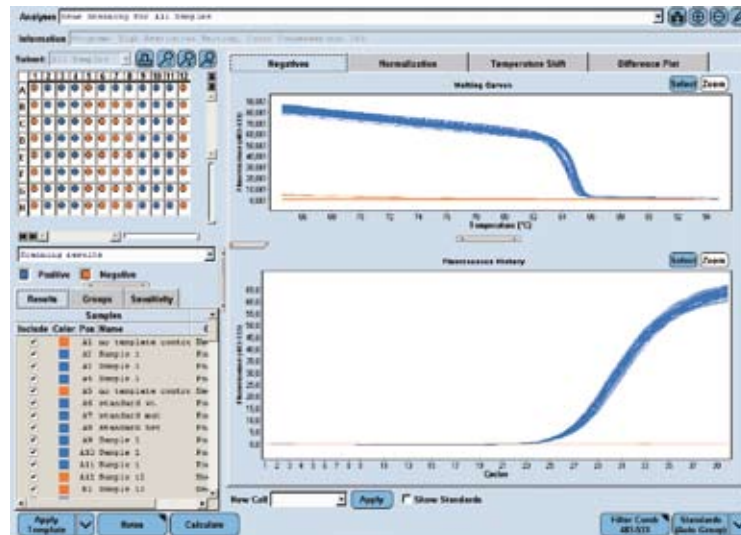




- The *Create new analysis* dialog opens. Select an analysis subset and an experimental program from the *Program* list (usually, you will select the melting curve program). If you wish, you can change the analysis name (default name = “*analysis type for subset name*”). Click .

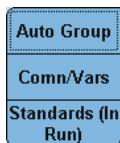





- The *Gene Scanning Analysis* screen opens.



**To perform a Gene Scanning analysis:**


- Using the *Standards* multi-select button, select the analysis mode (grouping method) you want to apply:



<p><b>Auto Group</b></p>	<p>Applies automated grouping in the absence of in-run melting standard samples.</p> <p> <i>If, in the Sample Editor, you have not defined any sample as a melting standard, the program will select this option by default after it creates the analysis.</i></p> <p>If the Auto Group analysis mode is selected, the software will assign all samples to a well-defined group (up to 6 variant groups can be assigned on the basis of melting curve shape); samples that cannot be assigned to such a group will be placed in either the Unknown or the Negative group.</p> <p> <i>Groups automatically assigned by the software are named with sequential numbers (1, 2, etc.). Some groups may contain no samples.</i></p>
<p><b>Comn/Vars</b></p>	<p>Applies automated grouping in the absence of in-run melting standard samples.</p> <p>If the Common/Variants analysis mode is selected, the software will place all samples into either group 1 (the largest group of samples with similar melting curve shapes, the so-called Common group), the Variants group, the Unknown group or the Negative group. No further grouping will be done.</p>
<p><b>Standards (In Run)</b></p>	<p>Applies grouping based on melting standard samples included in the run.</p> <p> <i>If, in the Sample Editor, you define samples as melting standards, the program will select this option by default after it creates the analysis.</i></p> <p><i>Groups based on internal standards are given names that correspond to the appropriate standards (assigned in the Sample Editor).</i></p>

**2 ▶ Deselecting Negatives**


If you wish, click the *Negatives* tab to designate samples as known negatives and remove them from the analysis (*i.e.*, deselect them).

-  *Although LightCycler® 480 Gene Scanning Software uses a negative filter to automatically deselect negative samples, it is still possible that all negative samples will not be detected. You will have another opportunity to correct this at the end of the run. You can do this by using the New Call option to assign known negative samples to the Negatives group (see below).*



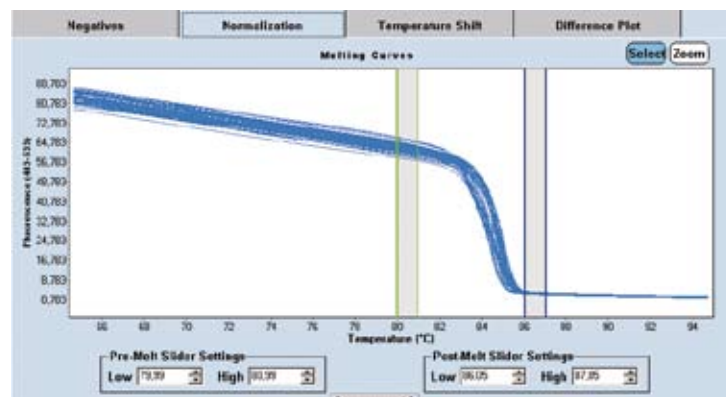
**3** ▶ **Normalization**

Click the *Normalization* tab to normalize the melting curves.

 *Fluorescence values acquired during High Resolution Melting will vary in magnitude, e.g., due to differences in the starting amount of template in each sample. This variability can mask differences between genotypes. Therefore the first step in the analysis process is to normalize the data. In normalization, two regions of each curve are selected (one before and one after the major transition); these are arbitrarily defined, respectively, as 100% fluorescence and 0% (baseline) fluorescence.*

The upper graph, the *Melting Curves* graph, contains two pairs of movable vertical sliders that correspond to *Pre-melt Low and High temperatures* (colored green) and to *Post-melt Low and High temperatures* (colored blue). The grey area between the sliders indicates the area used for normalization.

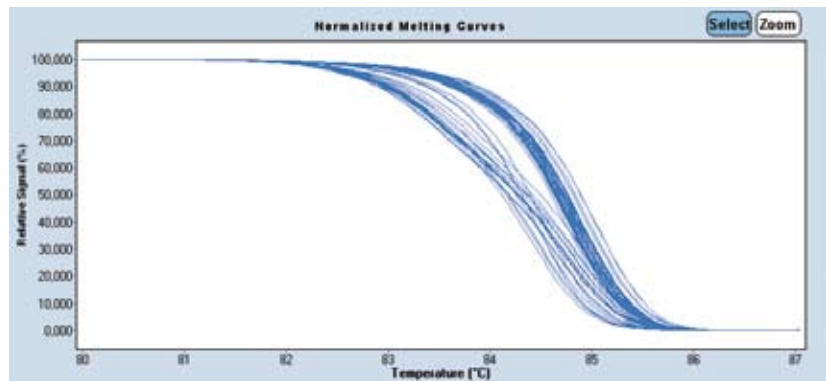
Below the Melting Curves chart you will see two groups of values that you can set: The left group is used to define the Pre-melt Temperature Range, while the right group is used to set the Post-melt Temperature Range.



- LightCycler® 480 Gene Scanning Software automatically places the temperature sliders in a suitable region for normalization and displays the normalized data in the lower graph (*Normalized Melting Curves*). Examine the upper graph to make sure that the *Pre-melt Temperature Range* lies in an area where the background fluorescence of all the samples is dropping consistently but no temperature transitions have occurred, and that the *Post-melt Temperature Range* is placed in an area where melting is complete for all samples.


If necessary, adjust the temperature settings by either dragging the vertical sliders on the upper graph or by changing the values in the *Slider Settings* fields. For most purposes, the recommended temperature interval between each set of vertical sliders is about one degree.

If the *Pre-melt* and *Post-melt Temperature Ranges* are set correctly, you should then obtain a set of normalized melting curves that have a flat section before and after the major transition; no curve should rise very far above the upper line or fall very far below the lower line.



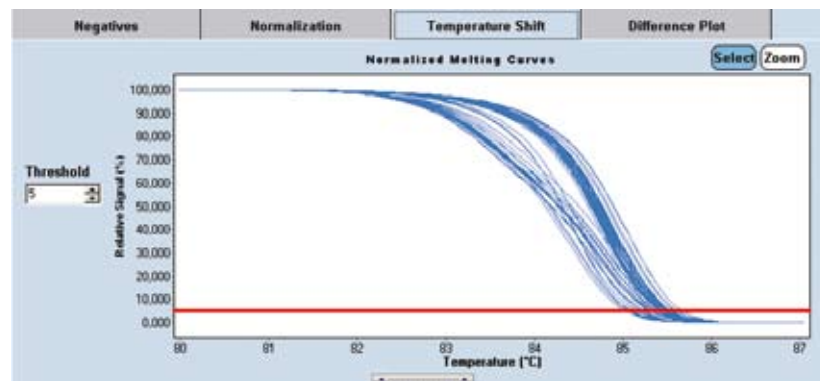
**5 ▶ Temperature Shifting**

Click the *Temperature Shift* tab to reset the temperature axis (x-axis) of the melting curves.

 *Eliminating the temperature offsets between samples can provide clearer separation of samples that have subtle changes in their melting profiles. The temperature axis of each curve is shifted in the region of low fluorescence (at the end of homoduplex melting) allowing heteroduplexes to be identified by their early drop in fluorescence.*

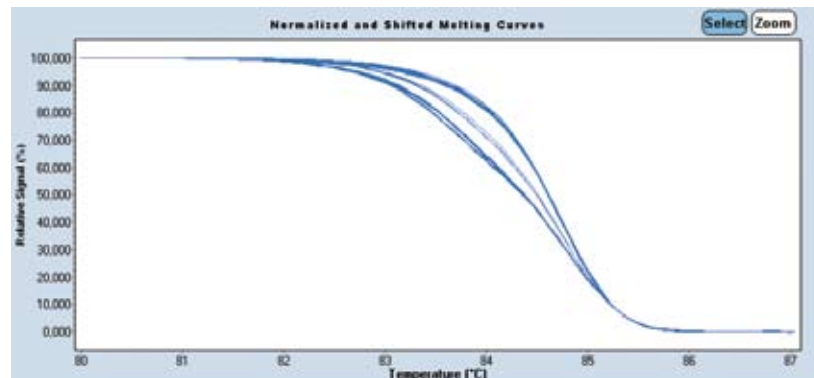
The display on the *Temperature Shift* tab contains two graphs: the upper graph is identical to the Normalized Melting Curves graph displayed on the *Normalization* tab, the lower graph shows melting curves that are both normalized and temperature shifted.

The upper graph has a movable horizontal slider that corresponds to the amount of the Temperature Shift. Left-hand of the graph you will see a control field, labeled *Threshold*, which can also be used to set the Temperature Shift level.



The default value of the Temperature Shift level is 5. You can change the value of the Temperature Shift either by entering a value in the *Threshold* control field or by dragging the horizontal slider on the upper graph.


Select a shift level that makes the curves form tight groups with the maximum amount of distance between groups (as shown in the image below). For most applications, the default level of 5 produces acceptable results.

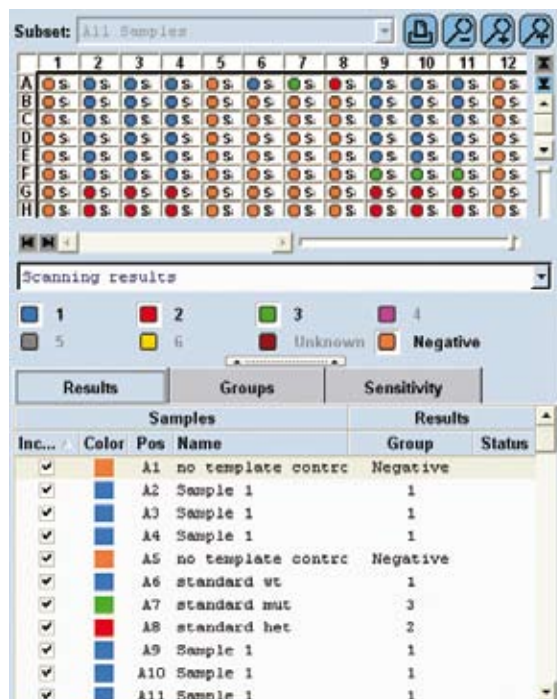


- 6 In the *Results* table, deselect any samples you do not want included in analysis of the results.
- 7 Now click the *Calculate* button to analyze the results and determine the grouping. The software determines variant (or genotype) groups and assigns a color and name to each group. Simultaneously, the software calculates the *Normalized and Temperature Shifted Difference Plot*.
- 8 Results of a Gene Scanning analysis include the *Sample Selector* with *Legend Property Selector* and *Legend Property* buttons and the *Results* table. Use the *Legend Property Selector* to display colors by result, by sample types, by sample preferences or by replicate groups. Use the colored *Legend Property* buttons to select the display of samples with certain properties in the MWP image, the *Results* table and in the charts.


If you choose *Scanning results* in the *Legend Property Selector*, the *Results* table of a Gene Scanning analysis displays the following results. The *Legend Property* buttons enable you to select samples depending on the variant group:

- ▶ Either 6 genotype groups (if you selected the Auto Group analysis mode) or 2 genotype groups (if you selected the Comn/Vars analysis mode).
- ▶ Negative and Unknown groups.

 Checkboxes will be shown even if the corresponding groups are not found in the data.



- ▶ Group: Name of the genotype group to which this sample is assigned.

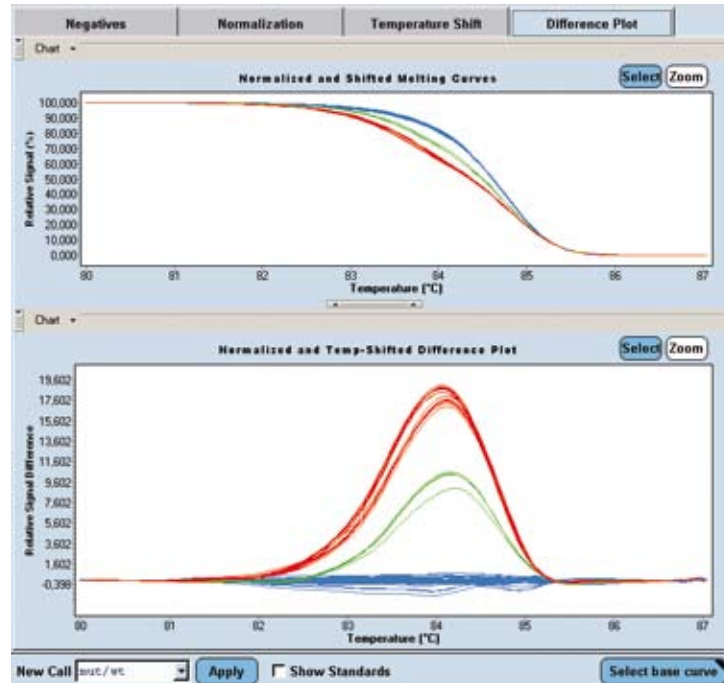
 Variant/genotype groups that are automatically assigned by the software are given sequential numbers (1, 2, etc.), while groups based on in-run melting standards are given names that correspond to the appropriate standards (assigned in the Sample Editor).



## 9 ▶ Difference Plot

Click the *Difference Plot* tab in the charts area to view


- ▶ the *Normalized and Shifted Melting Curves* and
- ▶ the *Normalized and Temperature Shifted Difference Plot*.



The Difference Plot is determined as follows:


- ▶ First the software selects the group that contains the most samples.
- ▶ Then the standard of that group (the Base Curve) is designated the reference genotype. The difference between the reference and each remaining curve is plotted against Temperature.

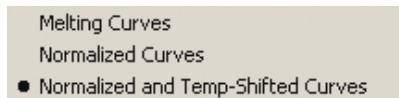
By checking the *Show Standards* box, you can also display the curves of the melting standards in the chart. These appear as black lines.

 *In Auto Group analysis mode, the median curve of each variant/genotype group is defined as the standard for that group.*


Click *Select base curve* to manually define the reference curve for the Difference Plot. By default, the standard of the largest variant/genotype is used as base curve.

### To customize the Difference Plot display:

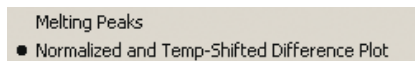
- 1 In the *Difference Plot* tab, click  either above the upper or lower chart. The chart's options toolbar is displayed, containing a chart menu.
- 2 To change the chart type, select the new chart type from the respective Chart menu. For the upper chart you have the following choices:



- ▶ Melting Curves: corresponds to the *Melting Curve* display in the *Negatives* tab, i.e., it shows the unnormalized and non-temperature shifted curves
- ▶ Normalized Curves: displays the normalized melting curves
- ▶ Normalized and Temp-Shifted Curves: corresponds to the *Normalized and Shifted Melting Curves* chart in the *Temperature Shift* tab.

 The normalized and shifted melting curves are basis for calculating the *Difference Plot*.

For the lower chart you have the following choices:

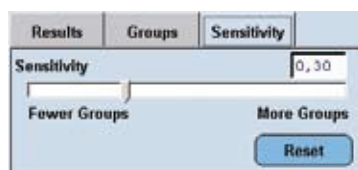


- ▶ Melting Peaks: displays the Melting Peaks plot (negative first derivative of the sample fluorescence plotted against temperature, corresponds to the result of the  $T_m$  Calling Analysis Module)
- ▶ Normalized and Temp-Shifted Difference Plot: displays the normalized and temperature-shifted Difference Plot (see Step 8 above)


### To change the sensitivity setting:


You can adjust the *Sensitivity* value to refine the Gene Scanning result. The *Sensitivity* function lets you influence the stringency with which melting curves are classified into different groups. A high *Sensitivity* value generally produces more groups than a low value.

Click the *Sensitivity* tab in the *Results* table to modify the analysis setting:



Slide the *Sensitivity* slider bar to the left to reduce stringency, or to the right to increase stringency.

 The default value is 0.3 (recommended for initial analysis). This value provides a reasonable balance between producing too many unknowns and generating potentially wrong calls.

 Please note, that in *LightCycler® 480 Gene Scanning Software* version 1.5 the sensitivity calculation has changed. This means that an analysis defined in an earlier version with a specific sensitivity value (e.g., "0,95") will not have the same results as an analysis defined in version 1.5 with the same sensitivity value.



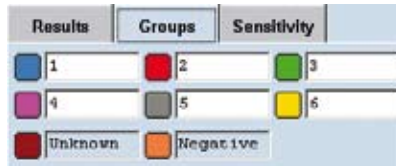
**To rename groups:**

On the *Groups* tab in the *Results* table, you can change the name associated with each variant/genotype group.

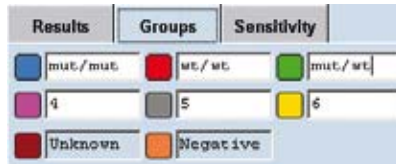


You can edit group names for groups generated by Auto Group analysis or generated by Comn/Vars analysis, not for groups based on in-run melting standards. Further, you cannot change the names of the Negative and Unknown groups or the number of group boxes that are displayed.

- 1 On the *Groups* tab, double-click the field for the group name you want to change.



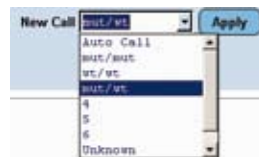
- 2 Type a new name in the field. The new name is immediately shown on both the *Sample Selector* and the *Results* table.



**To change variant/genotype calls:**

If you believe the software has assigned a sample to the wrong group, you can manually change the assigned group by using the *New Call* option.

- 1 In the *Results* table, select the samples to be changed. For details see the LightCycler® 480 Instrument Operator’s Manual, section “Working with Samples in the Analysis”.
- 2 From the *New Call* drop-down list below the chart area, select a new group name (genotype, Unknown, or Negative).



- 3 Click the *Apply* button.



- 
- 4 Selecting a new genotype group from the *New Call* list and clicking *Apply* changes all affected samples currently displayed in the graphs to the new name.

Selecting *Auto Call* from the *New Call* list (and clicking *Apply*) causes all affected samples currently displayed in the charts to revert to their previous (automatically determined) name.



*All manually modified calls are marked with an asterisk in the Results table and in reports.*



*You cannot assign a new call to negative controls. When in-run melting standards are used, you cannot assign a new call to them. If you attempt to apply a new call to a selection that includes an in-run melting standard or control, the software will warn you that the selection includes in-run standards or controls that cannot be changed, and asks you if you want to continue. If you choose to continue, the software will apply the changes to all samples except the in-run standards and controls. If you choose not to continue, the operation will be cancelled.*


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## E Supplementary Functions

### 1 Gene Scanning Template

A Gene Scanning analysis template contains the following settings:

- ▶ Subset and program
  -  *When you select a template, the software determines whether the current experiment contains a subset with the same name and the same well positions as the subset in the template.*
    - ▶ If the current experiment does not contain a subset with the same name, the software creates the subset.
    - ▶ If the current experiment does contain a subset with the same name, but the subset does not contain the same well positions as the subset in the template, the template cannot be used. You can delete the respective subset in the experiment, and then you are able to apply the template.
- ▶ Filter combination
- ▶ Analysis name
- ▶ Analysis mode setting (Auto Group, In-run Standards, or Common/Variants)
- ▶ Analysis notes
- ▶ Pre-melt and post-melt temperature settings
- ▶ Temperature shift setting
- ▶ Sensitivity setting

## 2 Result Control

The LightCycler® 480 Gene Scanning Software uses a control routine to check whether the analysis has passed or failed. The result control check is only applied to a run that contains internal melting standards; it is not applied to runs in *Auto Group* or *Conn/Vars* analysis mode.

- ▶ Negative Controls
  - ▶ If every Negative Control is designated negative, the run passes this control check.
  - ▶ If any Negative Control is not designated negative, the run fails this control check.
  - ▶ If any Negative Controls fail, the software will report no results and will inform the user that the Negative Control check has failed. The error dialogue also indicates which controls have failed this check.
- ▶ Melting Standards
  - ▶ If every internal standard is designated positive, the run passes this control check.
  - ▶ If all internal standards with the same group name are put into the same group, and standards with distinct group names are placed into distinct groups, the run passes this control check.
  - ▶ If any two internal standards with the same group name are not grouped together, the run fails this control check.
  - ▶ If any two internal standards with distinct group names are grouped together, the run fails this control check.
  - ▶ If any internal standard is designated negative the run fails this control check.

### 3 Unlicensed Features

The following statements define the behavior of the LightCycler® 480 Software when a gene scanning feature is used in the application, but a license for the LightCycler® 480 Gene Scanning Software is not available:

- ▶ An experiment that contains a Gene Scanning analysis can be imported into a system where the additional software module has not been installed. However, in this case, the Gene Scanning analysis and the analysis-specific Sample Editor will be set to read-only.
- ▶ When a macro that contains a Gene Scanning analysis is run on a system where the LightCycler® 480 Gene Scanning Software is not installed, the Gene Scanning analysis will not be performed.
- ▶ A template for a Gene Scanning analysis cannot be used on a system where the LightCycler® 480 Gene Scanning Software is not installed.
- ▶ When a report is generated from an experiment that contains a Gene Scanning analysis on a system where the LightCycler® 480 Gene Scanning Software is not installed, the report will contain the read-only information from the Gene Scanning analysis.

## 4 Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page, <http://www.roche-applied-science.com>, and visit our special interest site for

- ▶ the LightCycler® 480 System:  
<http://www.lightcycler480.com>
- ▶ The MagNA Pure System family for automated nucleic acid isolation:  
<http://www.magnapure.com>
- ▶ DNA & RNA preparation – Versatile Tools for Nucleic Acid Purification:  
<http://www.roche-applied-science.com/napure>
- ▶ Redefining Real-Time qPCR Assays with prevalidated UPL-probes:  
<http://www.universalprobelibrary.com>

Instruments		
LightCycler® 480 Instrument II, 96-well	1 instrument with control unit and accessories	05 015 278 001
LightCycler® 480 Instrument II, 384-well	1 instrument with control unit and accessories	05 015 243 001
Software		
LightCycler® 480 Software, Version 1.5	1 software package	04 994 884 001
LightCycler® 480 LIMS Interface Module	1 software package	05 066 310 001
LightCycler® 480 Gene Scanning Software	1 software package	05 103 908 001
LightCycler® 480 Multiple Plate Analysis Software	1 software package	05 075 122 001
Accessories		
LightCycler® 480 Thermal Block Cycler Unit (96-well) Silver	96-well thermal block cycler unit, including block cycler cover, storage box and loading device	05 015 219 001
LightCycler® 480 Thermal Block Cycler Unit (384-well) Silver	384-well thermal block cycler unit, including block cycler cover, storage box and loading device	05 015 197 001
Spare Parts		
LightCycler® 480 Dust Filters	4 filters	04 686 128 001
LightCycler® 480 Xenon Lamp	1 lamp	04 686 136 001
Disposables		
LightCycler® 480 Multiwell Plate 96	50 plates with 50 sealing foils	04 729 692 001
LightCycler® 480 Multiwell Plate 384	50 plates with 50 sealing foils	04 729 749 001
LightCycler® 480 Multiwell Plate 96, clear	50 plates with 50 sealing foils	05 102 413 001
LightCycler® 480 Multiwell Plate 384, clear	50 plates with 50 sealing foils	05 102 430 001
LightCycler® 480 Sealing Foil	1 x 50 foils	04 729 757 001
LightCycler® 480 Sealing Foil Applicator		04 706 170 001

<b>PCR Reagents</b>		
LightCycler® 480 High Resolution Melting Master	5 × 100 µl (500 reactions, 20 µl each)	04 909 631 001
LightCycler® 480 PCR Master SYBR Green	1 kit (5 × 100 reactions, 20 µl each)	04 707 516 001
	1 kit (10 × 500 reactions, 20 µl each)	04 887 352 001
LightCycler® 480 Probes Master	1 kit (5 × 100 reactions, 20 µl each)	04 707 494 001
	1 kit (10 × 500 reactions, 20 µl each)	04 887 301 001
	1 kit (1 × 5000 reactions, 20 µl each)	04 902 343 001
LightCycler® 480 Genotyping Master	1 kit (4 × 96 reactions, 20 µl each)	04 707 524 001
LightCycler® 480 RNA Master Hydrolysis Probe	1 kit (5 × 100 reactions)	04 991 885 001
<b>Labeling Reagents</b>		
LightCycler® 480 High Resolution Melting Dye	1 ml	04 909 640 001
SimpleProbe 519 Labeling Reagent	100 µmol	04 687 132 001
LightCycler® 480 CYAN 500 Labeling Reagent	1 vial (100 µmol)	04 764 153 001
LightCycler® Fluorescein CPG	1 g	03 138 178 001
	5 columns	03 113 906 001
LightCycler® Red 640-N-hydroxysuccinimide ester	1 vial	12 015 161 001
LightCycler® Red 610-N-hydroxysuccinimide ester	1 vial	03 561 488 001
LightCycler® 480 Demo Kit	1 kit (3 instrument runs)	04 710 924 001
<b>Isolation of Nucleic Acids</b>		
High Pure PCR Template Preparation Kit	100 purifications	11 796 828 001



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