LightScanner®
Operator's Manual

For Software Version 2.0

Designed for
Hi-Res Melting™

LightScanner®
Idaho Technology Inc.

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INSTRUMENT MANUAL SYMBOLS

The following symbols can be found throughout this manual. Use the definitions below as a guideline to their interpretation.

⚡	This symbol is used to indicate that non-compliance with instructions or procedures may lead to injury or even death.
♨	This symbol defines a potentially hot surface. Use caution when working near these areas to avoid bodily injury. Keep flammables and combustibles at a safe distance.
⚠	This symbol warns the user to operate the instrument according to the instructions provided in this manual. If used otherwise, a potentially hazardous situation could result.
🔍	This symbol highlights user tips to operate the instrument more efficiently.

ABBREVIATIONS

BSA..bovine serum albumin
CCD...charge-coupled device
cGMP...current good manufacturing practice
DHPLC ....denaturing high-performance liquid chromatography
DMSO...dimethyl sulfoxide
DNA..deoxyribose nucleic acid
DTT..dithiothreatol
EDTA ....ethylenediaminetetraacetic acid
HEPES ....4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid
HPLC..high-performance liquid chromatography
ITI ....Idaho Technology, Inc.
LED..light emitting diode
MOPS ....Tris, 3-[N-morpholino] propanesulfonic acid
NAGS ....N-acetylglutamate synthase
PCR..polymerase chain reaction
SAG ....small amplicon genotyping
SNP ....single nucleotide polymorphism
TGCE..temperature gradient capillary electrophoresis
TE ....Tris ethylenediaminetetraacetic acid
Tm ....melting temperature
TMSO ....tetramethylsulfoxide sulfolane
UNG..uracil-DNA glycosylase
UV/VIS...ultraviolet-visible

INTENDED USE

The LightScanner is designed to perform Hi-Res Melting™ of amplified nucleic acids. The resulting data can be used for mutation scanning or genotyping.

This instrument is intended for general laboratory use and should only be operated by trained laboratory professionals. Users are expected to have carefully read all the training material before beginning LightScanner operation.

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CHAPTER 1

INSTALLING THE LIGHTSCANNER INSTRUMENT

This chapter contains technical information about the LightScanner® instrument, including specifications, installation requirements, and installation instructions. It is included for people who install and maintain the LightScanner.

The LightScanner system consists of a single-temperature regulated heat block positioned under a high resolution charge-coupled device (CCD) camera. Polymerase chain reaction (PCR) is performed separately on a thermal block system before melting curve analysis on the LightScanner. The fluorescent DNA-binding dye (LCGreen® Plus) is included in the PCR reaction, eliminating post-PCR manipulation and reducing the possibility for amplicon contamination. A 96- or 384-well PCR plate is loaded into the LightScanner and analyzed with the help of the LightScanner software. The plate format you use depends on the LightScanner. Instruments configured for 96-well plates cannot be used with 384-well plates and vice versa.

Technical Data

Using the system improperly may compromise the integrity of the data, result in poor performance, or even permanently damage the equipment.

On delivery, please check that all listed items are included. If you notice any discrepancy or shipping damage, please contact Idaho Technology (ITI) immediately.

Components of the LightScanner System

The components of the LightScanner System are listed in the following table. Please note that these components may vary from country to country.

Component	Description
System Component 1	LightScanner Instrument 96-well plate format Catalog No. LCSN-ASY-0011 or 384-well plate format Catalog No. LCSN-ASY-0001.
System Component 2	LightScanner software package with CALL-IT® software and LightScanner Instrument Operator's Manual. Cable (serial) to connect LightScanner Instrument to the computer, cable connecting camera to computer, power cord (U.S.) or power cord (E.U.).
Computer Hardware*	2 X 512 MB RAM 80 GB hard drive, CD-RW drive, network card, keyboard, and PS/2 mouse.
Operating System	Microsoft® Windows XP Professional
Monitor*	17" Monitor with 1280 x 1024 graphics resolution

*Computer specifications may change

Instrument Specifications

Product	Specification
Size	7.8" (20 cm) width x 16.5" (42 cm) depth x 31.8 (81 cm) height
Weight	25 kg/55 lb
Sample format	Standard 96- or 384-well tray (white), reaction volume 5−100 μL
Data output	Plots melting profiles as fluorescence signal intensity vs. temperature.
LightScanner Software	Accurately performs mutation scanning and genotyping by Hi-Res Melting™ curve analysis.
Computer	Pentium-based PC with 17" monitor and software.
Networking	Ethernet connection allows export of data in standard spreadsheet format compatible with SQL*LIMS® software.
Voltage Input	100–240 VAC, +/-10%, 50−60 Hz with automatic detection and configuration, 2.5 A

Product	Specification
Wattage	800 W maximum including computer and monitor.
Excitation filter	470 +/-20 nm
Emission filter 1	510 nm
Emission filter 2 (optional)	600 nm
Excitation Source	Two sources illuminating target tray
Peak Excitation wavelength	470 nm
Sample temperature range	40 to 100 °C
Cooling time 95 to 60 °C (nonlinear)	~180 sec. at ambient temperature 25 °C
Heating rate	0.1 °C/s
Single run time	15 min., including cool down.

Safety Precautions for the LightScanner

⚠ Always observe these precautions when using the LightScanner Instrument:

- Follow all safety instructions printed on, or attached to, the analytical instruments.
- Observe all general safety precautions, which apply to electrical instruments.
- Never touch switches or power cord with wet hands.
- Do not open the housing of the LightScanner.
- Do not open the LightScanner during operation.
- Always wear safety goggles and gloves when dealing with toxic, caustic, or potentially infectious materials.
- When analyzing infectious material, use the LightScanner only in specially equipped rooms with controlled exhaust equipment, e.g., biological safety cabinets. Handling and disposal of infectious material should be performed according to local safety guidelines.

⚠ **CAUTION:** The heat block is hot while the instrument is operating.

💡 **Note:** Only authorized service personnel should perform service or repairs required for this unit.

Requirements for Installing the LightScanner

This section explains the requirements for placing, installing, and using the LightScanner instrument and the LightScanner computer.

Installation Requirements

⚠ Precautions for installation:

- Do not place the LightScanner next to instruments that cause electromagnetic interference or have high inductance (e.g., centrifuges or mixers).

- • Peripheral instruments connected to the LightScanner System must meet the IEC 950 (UL 1950) standard.
- • Use only the supplied power cord and RS 232 connector.
- • All plugs used in the LightScanner System (PC, printer, monitor) should have the same phasing to prevent switch-on peaks and electronic noise generated by other instruments or by the power supply itself. We recommend using a dedicated multiple-outlet surge protector for the LightScanner System.
- • Do not use the instrument in an explosive atmosphere.

Space and Power Requirements

Place the instrument in a site that can support the following instrument requirements:

Component	Specifications
Dimensions	The dimensions of the LightScanner are: 7.8" (20 cm) width x 16.5" (42 cm) depth x 31.8" (81 cm) height. At least 7" of clear space is required on the left side of the LightScanner instrument to provide easy access to the emission filter selection bar.
Weight	Approximately 55 lbs (25 kg)
Voltage Requirements	The LightScanner operates between 110 and 240 V (50–60 Hz). Make sure that a minimum voltage of 110 V is available for running the instrument. **Note:** The LightScanner adjusts automatically to the available voltage when the instrument is plugged in. The user does not have to set the instrument to the correct voltage manually. However, the PC does not automatically adjust to available voltage. The user must select appropriate voltage depending on the power source. Do not open the LightScanner housing.
Power Consumption	100–240 V, 50–60 Hz, 800 W maximum including computer and monitor.

Environmental Parameters

The instrument requires the following environment for accurate operation:

Description	Specifications
Ambient Temperature	15 to 35 °C
Ambient Temperature Required to Maintain Specifications During Operation	18 to 30 °C
Humidity	20–80%, no condensation
Altitude	Sea level to 2000 m

Storage Conditions

The LightScanner can be shipped and/or stored under the following conditions:

Description	Specifications
Ambient Temperature	-10 to +60 °C

Installing the LightScanner Instrument and Computer

🔵 **Note:** Transport the instrument by placing your hands under the base of the instrument. Position the instrument on the workbench. Allow 10 cm spaces to the left, right, and behind the instrument to insure sufficient cooling of the electronic components. Follow the procedures below to install the instrument and the computer successfully.

🔵 **Note:** We recommend connecting the LightScanner, PC, monitor, and printer to the same grounded power suppply.

🔵 **Note:** Ensure that PC and monitor have been set to the correct voltage. Do not place the instrument in direct sunlight or close to radiators or heating devices.

Air Exhaust

Indicator Lights

Emission Filter Slide

Air Intake

Sample Plate Slot

Serial Port

Bar Code Reader
(384-well system only)

Power Switch

Camera Port

Back and Front of the LightScanner

Power Cord

The LightScanner is provided with an IEC power cord equipped with a power plug that matches the destination country's standard power outlet or socket. The power cord has three pins: Live, Neutral, and Earth/Ground. Some outlets or sockets (especially in countries that use the CEE7/7 plug) may allow the Live and Neutral pins to connect and leave Earth/Ground pin unconnected. This should be avoided.

The Earth/Ground pin is provided for the safety of the user. Under fault conditions, the Earth/Ground connection can help prevent accidental electrical shock. For safety, please ensure that the Earth/Ground Pin has a reliable connection to the outlet's Earth/Ground pin and the outlet is properly wired. If in doubt, consult with the personnel in charge of the building's electrical facilities or have a certified electrician inspect the outlet.

IEC Cable with CEE7/7 Power Plug
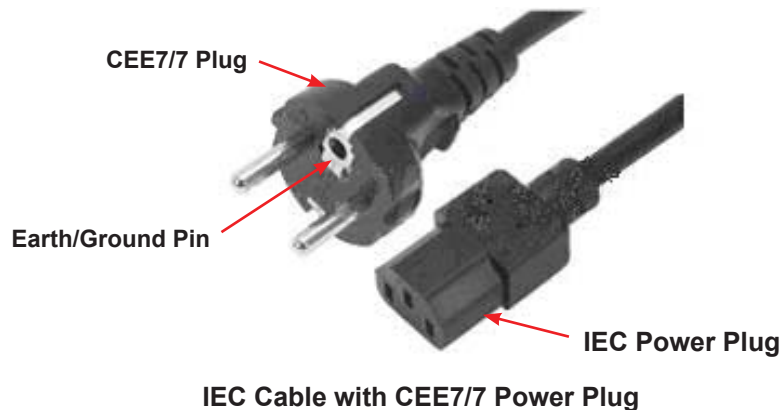
Connecting the LightScanner

1. Make sure the power is **OFF** on the LightScanner.
2. Connect the cables to the instrument (all on the back panel) in the following order:
   a. Power cord
   b. Camera cable
   c. Serial cable
3. Connect the camera cable to the computer tower (connection on the bottom of the tower).
4. Connect the serial cable to the computer.
5. Connect the remainder of the computer cables (monitor, mouse, power etc).
6. Next plug in the power cords from the LightScanner and computer to the wall power source.
7. Turn on the computer.
8. Turn on the power switch on the back of the LightScanner instrument. You will hear two beeps, the sound of a fan and the GREEN power light on the front of the instrument will turn on.
9. You are now ready to run the LightScanner.

Connecting the Computer with the Camera

When you first connect the LightScanner computer to the instrument you will see a message indicating that the computer has found new hardware. You will need to go through a simple 3-step wizard to allow the computer to begin communicating with the LightScanner camera. **Please follow the instructions below:**

Step 1

Step 2
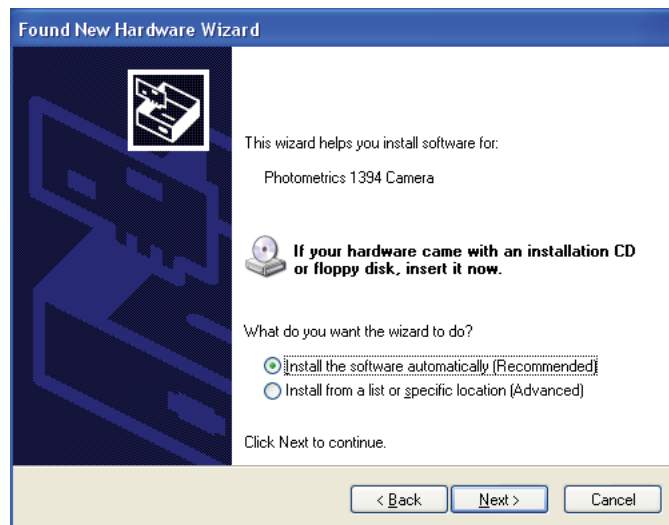
Step 3

The installation is now complete. Continue to the next step of the LightScanner setup, the Camera check (see manual for instructions).

Camera Check

A quick check to confirm that the camera is communicating with the computer should be performed.

Open the camera software found under:

Programs >> Photometrics >> RS config

1. A table will open displaying a NAME in the Camera 1 box, usually "***Camera 1***" confirming that the camera is communicating with the computer.
2. If there is not a name displayed and the boxes are gray, it indicates that the computer is not communicating with the camera. If this is the case, please contact Idaho Technology technical support.
3. Open the following program:

Programs >> Photometrics >> Pvcam test

4. Select **Collect Image.** This will generate an image of the heat block confirming that the camera is working correctly.
5. Once you see the image, click **Done**.

Calibration

After an instrument is installed, a plate position calibration will need to be performed. This calibration is performed in the **Run** window and requires a new, clean, completely empty plate.

1. Double-click on the **LightScanner icon** on the desktop.
2. Choose the **Run** tab.
3. Insert the empty plate into the instrument, notched edge first. The instrument should beep when the plate is loaded and the door closed.

4. Go to the **Tools** menu and select **Calibrate Map**.

5. An image of a plate with cross-hairs will appear. The cross-hairs can be moved using the mouse.

💡 **Note:** Instructions for calibration are also at the top of the screen.

a. First move the cross hairs over the center of the well at the top left corner (**A1**) making sure the cross is in the middle of the well (sometimes this is not the brightest part). Right-click and select **Enter**.

b. Next, repeat with the top right well (**A12**).

c. Lastly, repeat for the bottom left well (**H1**).

d. The software should produce an image of the plate where every well is ringed by a pink line.

e. Confirm that the positions of the wells are not skewed.

6. If you are not happy with the results of the calibration, repeat with a different plate. Sometimes adhesive material from the film may produce pink streaks on the lip of a well. If this happens, repeat the calibration using a different plate.

Calibrate in the following order
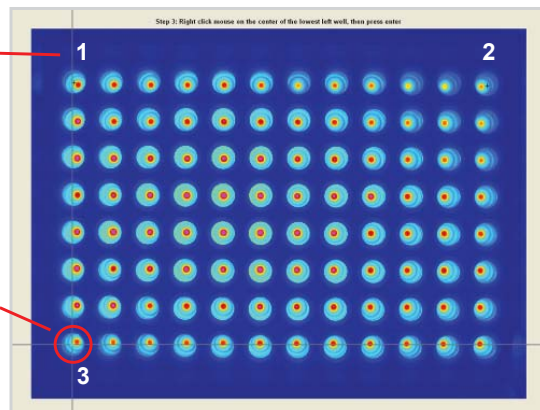
Cross hatch is over the center of the circle, and not on the darkest part of the well

When finished, the pink circles need to be centered around each well. This image shows a good example of a calibrated plate

7. Calibration should be repeated if the instrument is moved to a new location.

8. Weekly or monthly recalibrations are not necessary. It is only to be done the first time the instrument is set up or after it has been moved.
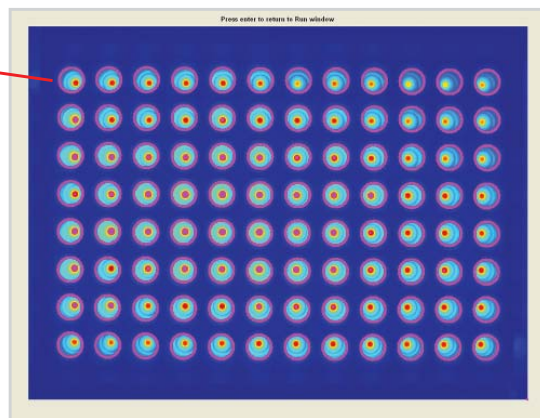
LightScanner Instrument Operation

The LightScanner consists of a temperature-regulated heat block positioned under a high-resolution CCD camera. PCR is performed in 96- or 384-well format plates on a thermal block before melting curve analysis on the LightScanner. The plate is then loaded into the sample plate slot of the instrument. A stepper motor lifts the plate to a precise location so that a uniform focal length is maintained. Banks of LEDs excite the fluorescent dye in the plate wells. The temperature in the block is then slowly and consistently increased. A structural grid ensures the sample plate will not warp during this slow heating. The high-resolution CCD camera captures fluorescence and the data are transferred to the computer for analysis by the LightScanner software. A built-in bar code reader provides the option of sample tracking and documentation as necessary.

LED Indication Lights

Three indicator lights are located at the front of the LightScanner. All of the lights come on when the instrument is switched on. During instrument operation, the lights function as described in the table below:

Position of Light	Color of Light	Label	Function	Indication
Top	Green	Power on	On	Instrument is switched on
			Off	No power, instrument is defective
Middle	Red	Running	On	Instrument is running
Bottom	Yellow	Run Completed	On	Instrument is not running either because the run is completed or because the run has not yet been started. Plate can be safely removed.
			Off	Instrument is running.

Cleaning and Decontamination

🛈 **Note:** Before cleaning, ensure all electrical plugs are disconnected.

Clean the external parts of the instrument with a sponge moistened with the minimal amount of water containing a mild detergent. The emission filters can be wiped free of dust using a clean, dry, lint-free optical cloth.

If spills or leaks occur inside the heat block area, please contact an ITI representative. Do not attempt to disassemble the LightScanner to gain access to this area.

CHAPTER 2

HI-RES MUTATION SCANNING

High Resolution Melting (Hi-Res Melting™) of nucleic acid depends on the ability to collect high-density information of fluorescence as a function of temperature in a mixture that contains a fluorescent double-strand DNA binding dye LCGreen Plus and PCR product. In the LightScanner, images of DNA melting (i.e., fluorescence) are captured by a CCD camera and magnified to reveal subtle details in DNA melting profiles. Sample-to-sample comparisons of these images are then used to interrogate the sequences of the amplified DNA. Correct interpretation of the data depends to a large extent on the software algorithms that are used. LightScanner software has been developed specifically to provide the most accurate analysis of Hi-Res Melting curves.

This section will cover the basic concepts of Hi-Res Melting and mutation scanning using LCGreen Plus dye.

Overview of Hi-Res Melting for Mutation Scanning

Screening amplified DNA for sequence variation, also known as "mutation scanning", is an important tool for genetic research and clinical applications. The LightScanner is unique in that it allows **homogeneous mutation scanning** in standard micro-titer format, using a saturating dsDNA binding dye and Hi-Res Melting.

Mutation scanning techniques detect the presence of sequence variation in a fragment of amplified DNA. With a diploid genome, equivalent PCR fragments are generated from maternal and paternal chromosomes. The DNA fragments are analyzed for completely matched hybrids called **homoduplexes**, and mismatched hybrids called **heteroduplexes**. Conventional scanning techniques are not homogeneous and require a separation step to identify heteroduplexes.

Mutations in PCR products are detected by changes in the shape of the melting curve compared to a reference sample. Below are examples of single nucleotide polymorphisms (SNPs) identified in two exons using the LightScanner instrument and our LCGreen Plus Dye. Superior reproducibility is demonstrated by the overlapping curves of duplicate samples for both the wild type and the mutant samples.
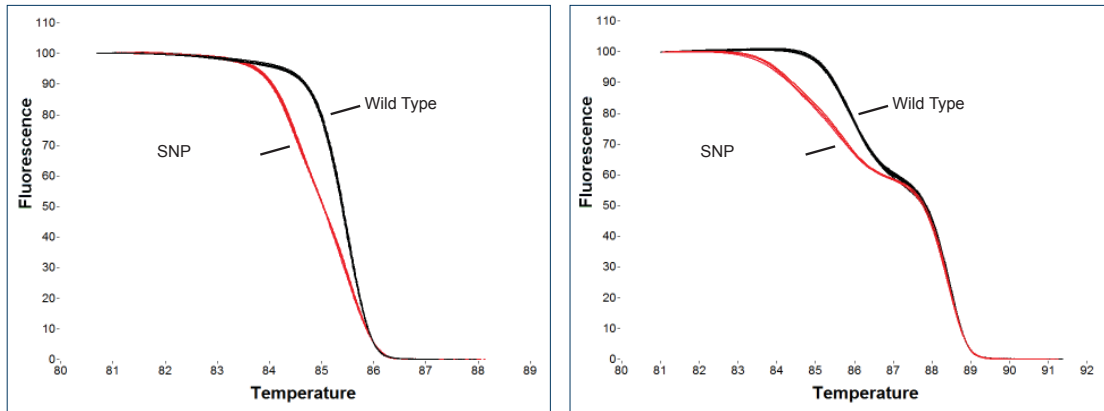
The LightScanner uses the fluorescence of a new category of dsDNA binding dyes, the LCGreen dyes, to identify sequence variations without the need for dye-labelled probes. The system requires no post-PCR addition of reagents or the need for separation. The LCGreen Plus dye is included in the amplification reaction, and a Hi-Res Melting profile, requiring only 2–5 minutes, reveals sequence variations.

Hi-Res Melting analysis with the dye LCGreen Plus identifies heterozygous single-base changes in PCR products with a sensitivity and specificity comparable or superior to non-homogeneous techniques. Variants are detected independent of their position within the fragment. Sensitivity is highest in fragments 400 bp or fewer but scanning has been successfully performed with fragments up to 1000 bp. In addition to identifying anonymous heterozygous variants, Hi-Res Melting can often identify the specific mutation, in such cases scanning and genotyping can often be combined into one simple melting analysis. A more difficult application is the identification of homozygous sequence alterations; most other mutation scanning methods are unable to distinguish these alterations from wild type sequences. By contrast, Hi-Res Melting has been shown to be capable of identifying homozygous sequence alterations in several different types of amplicons. Certainly, not all homozygous changes can be detected this way, but many will be.

Why LCGreen Plus Works

Hi-Res Melting analysis relies on a new family of dsDNA binding dyes called LCGreen. The unique feature of LCGreen is the ability to detect the presence of heteroduplexes formed during PCR if the sample is heterozygous for a particular mutation. This feature is not shared with other dyes traditionally used in real-time PCR, such as SYBR Green or ethidium bromide.
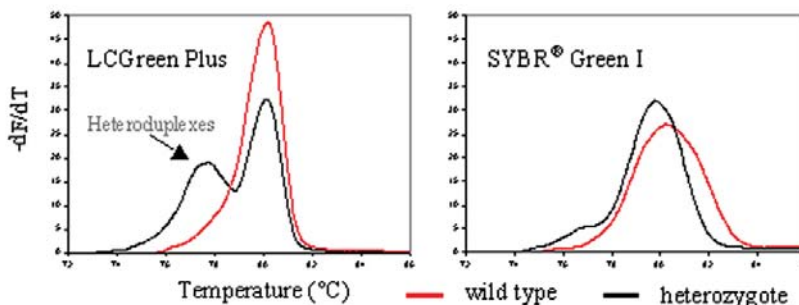
Derivative melting curves illustrate detection of heteroduplexes in the heterozygous mutant using LCGreen Plus which are not detected using SYBR Green I.

Data Analysis Basics for Hi-Res Melting

The power of Hi-Res Melting is augmented by analytical software that allows visual inspection of melting curve data to identify changes in the shape of the curve, indicating the presence of sequence variances in the PCR product.

Step 1. The first step in the analysis is to **normalize** the raw melting data (wild type samples are shown in black; and samples with SNP alleles are shown in other colors). In some cases, samples with homozygous SNPs may be distinguished from the wild type by shifts in melting temperature.
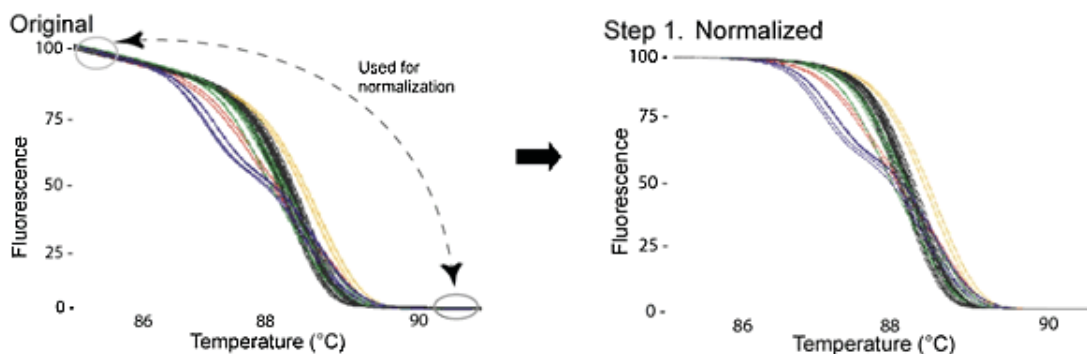
Step 2. The next step is to **temperature shift** the curves at the point where the entire double-stranded DNA is completely denatured. Now, samples with heterozygous SNPs can be easily distinguished from the wild type by the shift in the shape of their melting curves, which often cluster into distinct groups according to genotype.
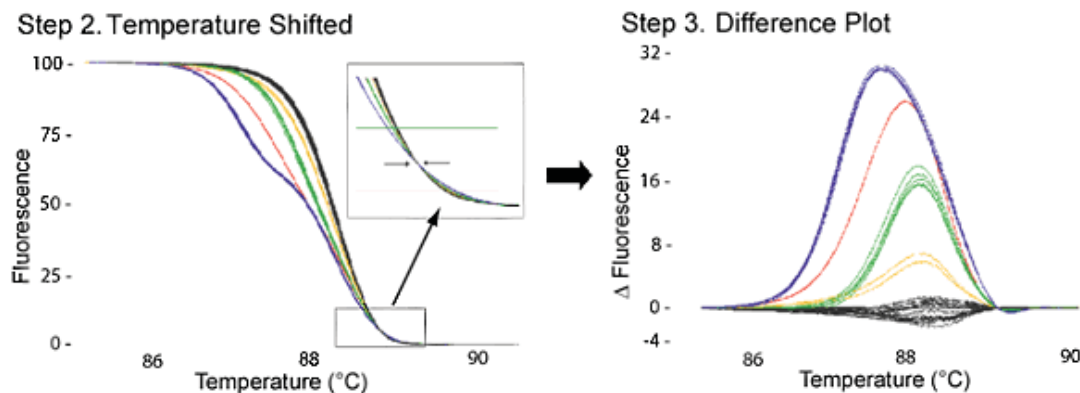
Step 3. The final step is to further analyze the shape differences by subtracting the curves from a reference curve, thus generating a **Difference Plot**, which helps cluster samples automatically into groups using the Call-IT software.

Scan Before You Sequence

Hi-Res Melting can identify sequence variants in an amplicon, but it is generally recommended that the variants are confirmed by sequencing. The LCGreen Plus dye that is added to the PCR mixture is compatible with traditional sequencing methods and will not damage the post-PCR product.

If an amplicon is known to contain a common polymorphism, the presence of the common SNP can be ruled out by second-tier testing with an unlabeled probe instead of sequencing. The LightScanner optics are designed to be compatible with Idaho Technology's SimpleProbes and a genotyping module in the software allows the user to perform scanning and probe based genotyping on the same platform.

For more information on the LightScanner and its applications please visit our web site at **www.idahotech.com.**

CHAPTER 3

PREPARING A LIGHTSCANNER EXPERIMENT

Before you begin a LightScanner experiment, you must amplify your sample DNA using PCR in the presence of LCGreen Plus. After DNA amplification, you can immediately place the sample plate into the LightScanner and begin Hi-Res Melting. No post-PCR sample manipulation is required.

To generate the best results, it is essential to optimize the DNA amplification such that nonspecific targets are minimized (or eliminated altogether) and that the desired product is robust.

This section provides step-by-step instructions for preparing a plate for a combined PCR and LightScanner run. Plate specifications are different depending on whether you are using a 96- or 384-well PCR plate. For information about using the LightScanner software to program the LightScanner instrument and start the run, see Chapter 4, Running a LightScanner Experiment.

Optimizing PCR for Hi-Res Melting

General Considerations and Tools Required for Assay Development

Modern laboratories are equipped to generate and optimize PCR products for application to Hi-Res Melting. To successfully execute Hi-Res Melting assays, the following are necessary: 1) Laboratory-hardware, 2) Software, 3) Reagents, 4) Sample preparation, 5) Oil overlay, and 6) PCR optimization. Each of these issues is first considered separately followed by how they act in concert to develop a gene-scanning assay.

Hardware

- LightScanner Instrument
- Thermal block PCR instrument (an instrument with temperature gradient function is beneficial)
- Plastic disposables (see recommendations in Appendix B)
- Gel electrophoresis system
- Ultraviolet-visible (UV/VIS) spectrophotometer

- Standard laboratory equipment (pipettes, centrifuge, consumables, etc.)
- LightCycler® (optional)
- Hi-Res Melting Instrument, HR-1™ (optional, Part No. HR01-ASY-0001)

Oligonucleotide Design Software
Oligonucleotide design software is an indispensable tool for the modern molecular biologist; however, software is only a tool that suggests appropriate primer design. The most powerful capability of software is identifying undesired base pairing interactions such as hairpins, homodimeric interactions, heterodimeric interactions, and primer to amplicon interactions. Unfortunately irrelevant interactions are often identified that will never form in PCR and exist only in computer simulation. There are numerous primer design software packages, each having characteristic strengths and weaknesses. We recommend using the LightScanner Primer Design Software for designing primers to be used with the LightScanner System.

Reagents
Use of the LightScanner Master Mix will drastically reduce the amount of time required to optimize primer sets. Use of LCGreen Plus in combination with other commercially available PCR buffers is possible but may require additional optimization. Please see Appendix E, Additional Optimization Considerations, for more details.

Sample Preparation
Hi-Res Melting compares profiles from independent PCR reactions; therefore, minimizing reaction-to-reaction variability is pivotal. Samples to be analyzed using Hi-Res Melting should be extracted using a common extraction platform. This eliminates the subtle differences in the reagent components between the final elution buffers from multiple extraction platforms.
Ten to 20 ng of template genomic DNA in a 10-µL reaction is recommended.

Oil Overlay
To avoid evaporation of the samples during Hi-Res Melting an overlay is necessary. The recommended overlay is common mineral oil. For a 10-µL PCR reaction, a 15–20 µL mineral oil overlay is required.

PCR Optimization
Specific amplification product, undesired amplification product(s), and dimer product all bind LCGreen Plus with equal affinity and contribute to overall fluorescence and the melting profile. To optimize the amplification determine the best concentration for individual reaction components and the best thermal cycling parameters. The steps below focus upon developing a new PCR reaction; however, these may be used to adapt established PCR reactions to Hi-Res Melting.

Note: In thermal cycling, the annealing temperature influences specificity and robustness of amplification the most. Gradient PCR is useful for quickly optimizing PCR cycling conditions.

Step 1: Set up the PCR reaction using the LightScanner Master Mix. The following reaction volumes are recommended in a final reaction volume of 10 µL.

Component	Volume	Final Concentration
LightScanner Master Mix	4 µL	1X
Forward Primer	1 µL	0.25 µM
Reverse Primer	1 µL	0.25 µM
Water	3 µL	NA
DNA	1 µL	10–20 ng

Step 2: Program a gradient-capable thermal block with the following protocol to determine optimum annealing temperatures (for primers with estimated Tm's of 60–64 °C).

Cycles	Temperature	Time
Hold	95 °C	2 min.
	94 °C	30 sec.
45 cycles	60–72 °C	30 sec.
1 cycle (heteroduplex formation)	94 °C	30 sec.
	25 °C	hold until plate is removed

Step 3: Run the samples on the LightScanner Instrument using the following parameters:

Start Temperature	70 °C
End Temperature	96 °C
Hold Temperature	67 °C
Exposure	Auto

Step 4: Load 7 µL of each sample onto a 2–4% agarose gel for analysis.

The combination of the PCR information and the resulting melt data provide a method for determining the optimal PCR conditions.

Choosing the Best PCR Condition

Correlate the melt profiles of the gradient experiment plate with the gel results to choose the best candidate PCR condition. Use the following guidelines to interpret the data. See the sample gel image and melt profile below.

1. Identify the highest annealing temperature where the desired product is created (this will likely be a less robust band but should be the only visible band in that sample).

2. Identify the lowest annealing temperature where specificity of the primers is maintained (this should be one of the most robust bands on the gel).

3. The annealing temperatures in between these temperatures (highest and lowest) are all candidate PCR conditions for the primer set.

4. Analyze the melt profiles of the candidate PCR conditions identified from the gel.

·   The condition that has the greatest amount of initial fluorescence combined with the greatest amount of fluorescence change from the beginning of the melt to baseline is the best candidate.

In the following example, an annealing temperature of 70.1 °C is optimal.
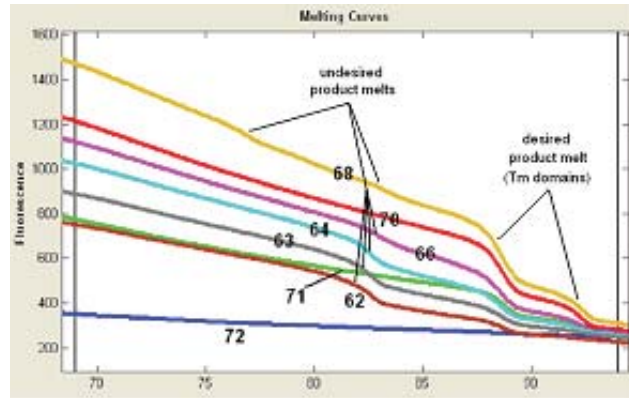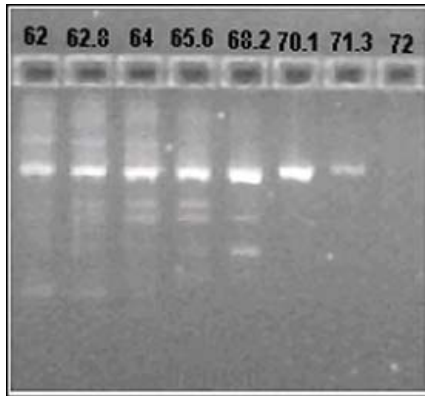
Candidate PCR Condition Duplicate Experiment

Using several (at least 10) different DNA samples, all from a common DNA prep, run each sample in duplicate using the best candidate PCR protocol based on the gradient experiment.

Melting curve analysis on the LightScanner will usually be sufficient for determining the success of the duplicate plate experiment.

*   All samples should amplify robustly
    –   Auto exposure should be ≤ 80ms
    –   Initial fluorescence in all samples should be greater than 800

*   All samples should have "clean" melts
    –   No small melts at lower than expected temperatures based on the melting results of the gradient experiment plate

*   Duplicate samples should group together

*   All samples should group easily into a common group
    –   Unless there are common variants in the fragment, or samples with known variants were run on the duplicate plate experiment
    –   If samples with known variants were run, these samples (and their duplicates) should group separately from the common, WT group.

*   If excessive manipulation of the normalization cursors or temperature shifting threshold is necessary to get the sample curves to group, further optimization may be beneficial.
    –   MgCl titration (2.0–3 mM final)
    –   Primer concentration (0.15–0.4 µM final),
    –   DMSO (5-10% final) or TMSO (0.5–1% final)

If further optimization efforts are not successful on a new annealing temperature gradient experiment, the primers may require redesign.

Special Considerations for Assay Development for Genotyping with LunaProbes™

Method Background

Genotyping with unlabeled probes is an homogeneous, end-point assay. The probe is included in the PCR mix but is not consumed during amplification. Genotyping is accomplished by monitoring the melting of probe-target duplexes post-PCR. Key to the method is the use of one primer in excess, which leads to the overproduction of the target strand.

Assay Design

Use the following guidelines when designing an unlabeled probe genotyping assay:

*   Identify the location of the SNP and determine all the variants present in the region of the amplicon to be sure that you do not place your primers across any polymorphisms.

*   Design primers with a Tm of 60–65 °C.

*   Design the probe with a Tm of 65–70 °C (we recommend designing the probe an average of 5 °C greater than the primer Tm).

*   Design probe length to be between 20 and 35 bases.

*   Design the probe with the mutation site located towards the middle of the probe to maximize the instability with the mismatched variant.

*   To prevent extension of the probe, a C3 block is recommended for the 3' end. (3' C3 blocking is available from most oligonucleotide synthesis vendors; specify when ordering the probe).

*   Check primers and probes using commercial design software to minimize complementarity, hairpins, etc. Check oligos using the Basic Local Alignment Search Tool (BLAST) to ensure specificity.

Experimental Set Up

Reaction Chemistry: 10 µL per reaction volume

Reaction Component	Volume (µL)	Final Concentration
LightScanner Master Mix	4	1X
Forward Primer	1	0.1 µM*
Reverse Primer	1	0.5 µM
Unlabeled Probe	2	0.5 µM
DNA	2	10 ng

*Note the 5:1 asymmetry in primer concentration, favoring formation of the probe-target duplex.

Cycling Conditions

Initial Denaturation	94 ºC 30 sec.	1 cycle
Amplification	94 ºC 30 sec. 64 ºC 30 sec. 72 ºC 30 sec.	55 cycles*

*55 cycles of PCR is recommended to allow, on average, 10 cycles of amplification beyond the plateau phase of the PCR reaction to ensure an abundant quantity of probe/target complex.

Genotyping Results—Example

A single probe recognizes all three genotypes. In this example, the probe is designed to match the G allele. Probe-target hybrids to the G allele have a higher melting temperature because they are perfectly paired. The C allele hybrids have a lower melting temperature due to the probe-target mismatch. The heterozygous samples (shown below in red) demonstrate melting profiles resembling the combination of homozygous samples.
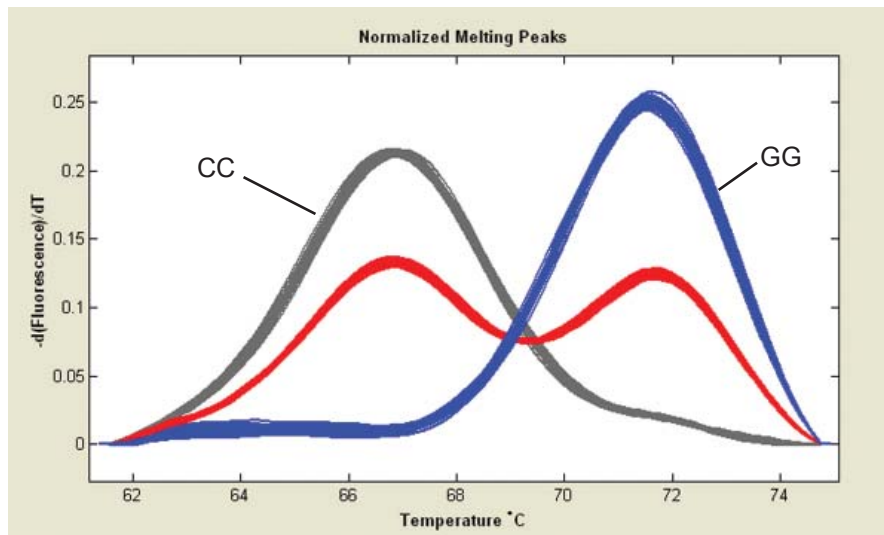
For more information on PCR optimization, optimizing internal reaction chemistry, developing a panel of PCR assays to function together in Hi-Res Melting, and secondary uses of PCR product previously employed for Hi-Res Melting, see Appendix E, Additional Optimization Considerations.

CHAPTER 4

RUNNING A LIGHTSCANNER EXPERIMENT

The LightScanner software controls the LightScanner instrument based on information you pro-vide, such as the starting and ending instrument temperatures.

This chapter explains the preparations you must make before running the experiment, then describes the experiment steps in detail.

Preparing a Plate for a LightScanner Experiment

1. Prepare a master mix containing all the reaction reagents except the DNA/RNA template in a 1.5 ml or 0.5 ml reaction tube.
2. Pipette oil overlay into each well.
3. Pipette the reagent mix into the wells on the PCR plate. Robotic or parallel (8- or 12-tip) pipetting is helpful when processing large numbers of samples.
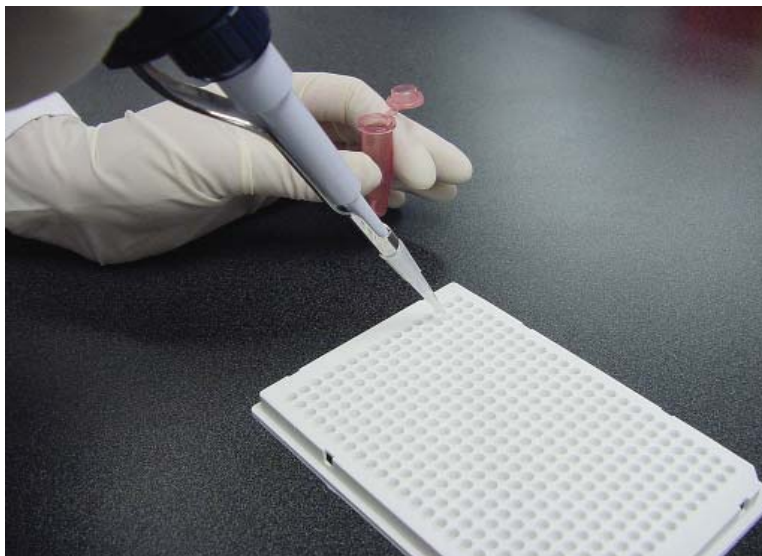
4. Add the DNA template to each well.

5. Seal the plate with adhesive plate film.
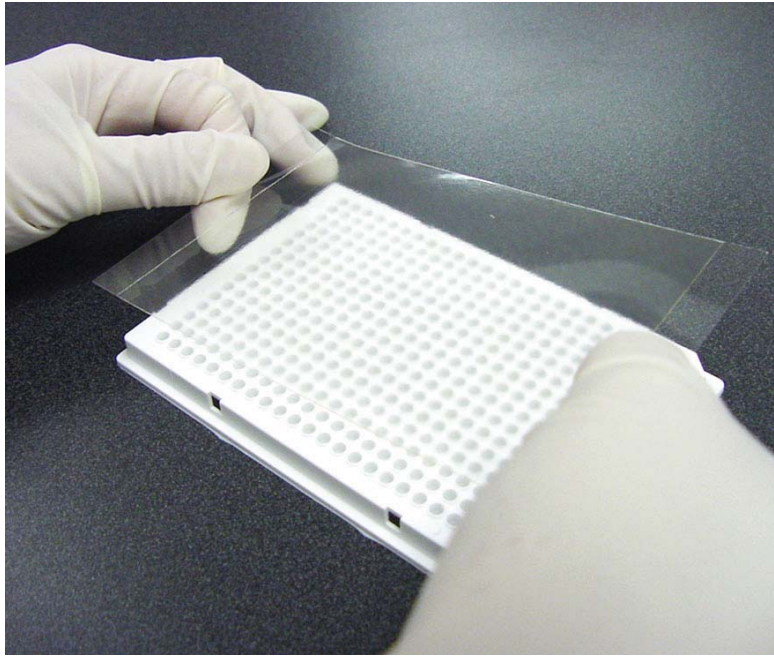
6. Centrifuge plate at 1000 rpm for 1 min. Check the wells for bubbles and repeat if necessary.

Note: Centrifuge only with appropriate plate adapters. Do not exceed a centrifugation speed of 3000 rpm.

7. Perform PCR amplification on a thermal block cycler.

💡 **Note:** Thermal block protocol should end with a final denaturation step (hold for 30 sec. at 94 ºC), followed by rapid cooling to a final hold at 20 °C.

8. Repeat centrifuge step.
9. Remove adhesive film from plate if not using optically clear tape.

Running an Experiment

- Turn on the LightScanner instrument and computer.
- Double-click the LightScanner icon on the computer desktop to start the software.

⚠️ **WARNING:** Do not turn the instrument off while the software is running. If you turn the instrument off, you must close the software, restart the instrument and the software, then restart any experiment that was in progress.

A message bar in the "Run Status" box displays the current status of the instrument ("Ready"). During the experiment run, the message bar displays the current temperature and estimated time to completion. The message bar also displays instructions for various tasks, as necessary.

1. Select **New Run** from the file menu or press the **Run** button at the top of the screen.
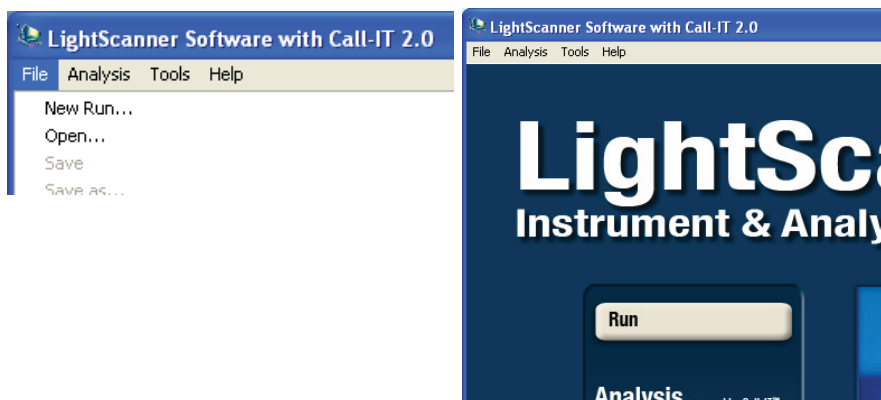
Setting the Starting and Ending Temperatures

When you start an experiment, you must specify the starting and ending temperatures for the melt. The default temperature parameters are 45 °C and 90 °C. If you are confident of the amplicon's melting temperature you can adjust these temperatures accordingly.

⚠ **CAUTION:** If you set temperature parameters too close to the melting temperature range of the amplicon you may not detect melting transitions associated with unknown polymorphisms. Be conservative with your initial estimates. Enter a temperature range wide enough to encompass any melting temperatures associated with the formation of heteroduplexes.

To Set the Temperatures

1. In the **Start Temp** and **End Temp** boxes, select the starting and ending temperatures for the melt.

2. The **Hold Temp** box will automatically be set to 3 °C below the start temperature.

3. Leave **Auto** selected in the **Exposure** box. (For information about manually setting the exposure, see "Manually Determining Exposure Time," later in this chapter.)

4. If you use a bar code, click **Bar Code** once, then hold the side of the plate with the bar code sticker in front of the bar code reader on the LightScanner (bar code reader only available on 384-well LightScanner).

   The red bar code reader light turns on. When the bar code is scanned, the instrument issues a beep, then the light turns off. The bar code is displayed in the message bar.

🔵 **Note:** If you make a mistake, for example scanning the wrong bar code, you must start a new experiment before you can rescan the bar code.

Loading a Plate and Starting the Run

⚠ **CAUTION:** Use only white PCR plates in the LightScanner instrument (see Appendix B).

1. When using a 96-well LightScanner with a half-skirt plate, set the plate into the provided adapter before inserting the plate into the machine.
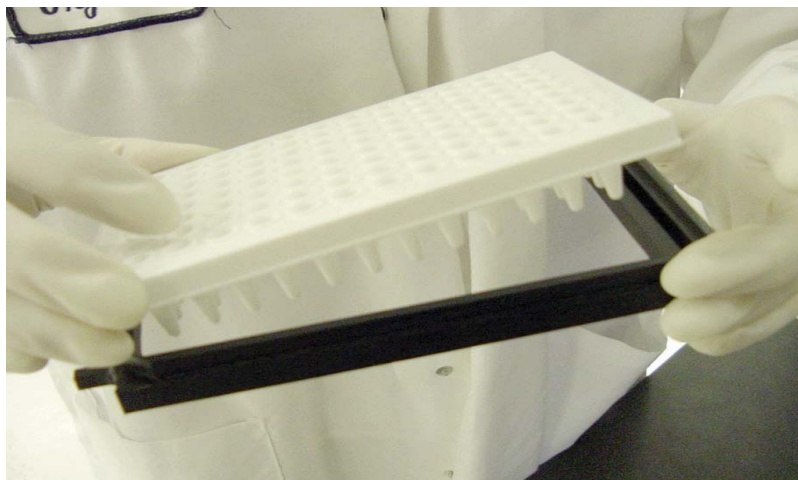
2. Load plate into LightScanner, inserting the notched edges first. Push the plate fully into the LightScanner. When the machine detects the plate, it issues a beep and automatically closes the door.

3. Click **Start Run**

4. If a bar code was scanned before inserting the plate, the software appends the name of the machine and the current date and time to the bar code. The experiment files will have the following name format:

barcode_machine_date_time.extension

5. If a bar code was not scanned, a file selection dialog will be displayed. Select an experiment name used to save the collected data and press **Save**.
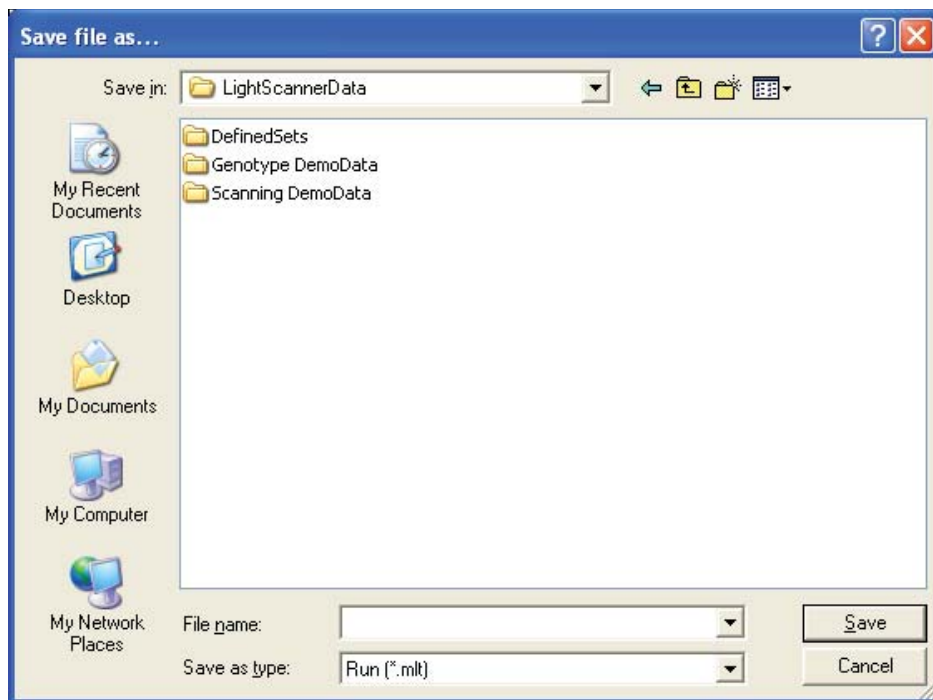
The software appends the name of the machine and the current date and time to the experiment name. The experiment files will have the following name format:

alphanumeric-name_machine_date_time.extension

The software creates a folder with the same name as the experiment and stores all experiment files in the folder. The default location for the folder is C:\LightScanner Data\. (If you enter an experiment name, instead of a bar code, you can choose to save the experiment data in another location.)

Note: If you give the experiment the same name as a previous experiment, the associated files are saved in the previously created folder. In this case, the unique identifier for the file names is the date and time stamp.

Note: If the current temperature of the instrument is greater than the designated starting temperature, you are prompted to turn on the instrument fan. Click **Fan On**. The tray is ejected. The message bar displays temperature readings as they decrease. When the temperature reaches 3° C below the designated starting temperature, the message bar says "Ready." Rescan the bar code (if using), reinsert the plate, and click **Start Run** to restart the experiment.

During the Run

Once the run starts, the LightScanner determines the proper exposure, fine-tunes the well map, and then starts collecting the scanning data. Messages in the message bar describe the instrument's progress.

When the block reaches the starting temperature, the message bar displays the current instrument temperature and estimated time to completion. The **Run Status** box displays the run name, the run parameters, and the message "**Started**."
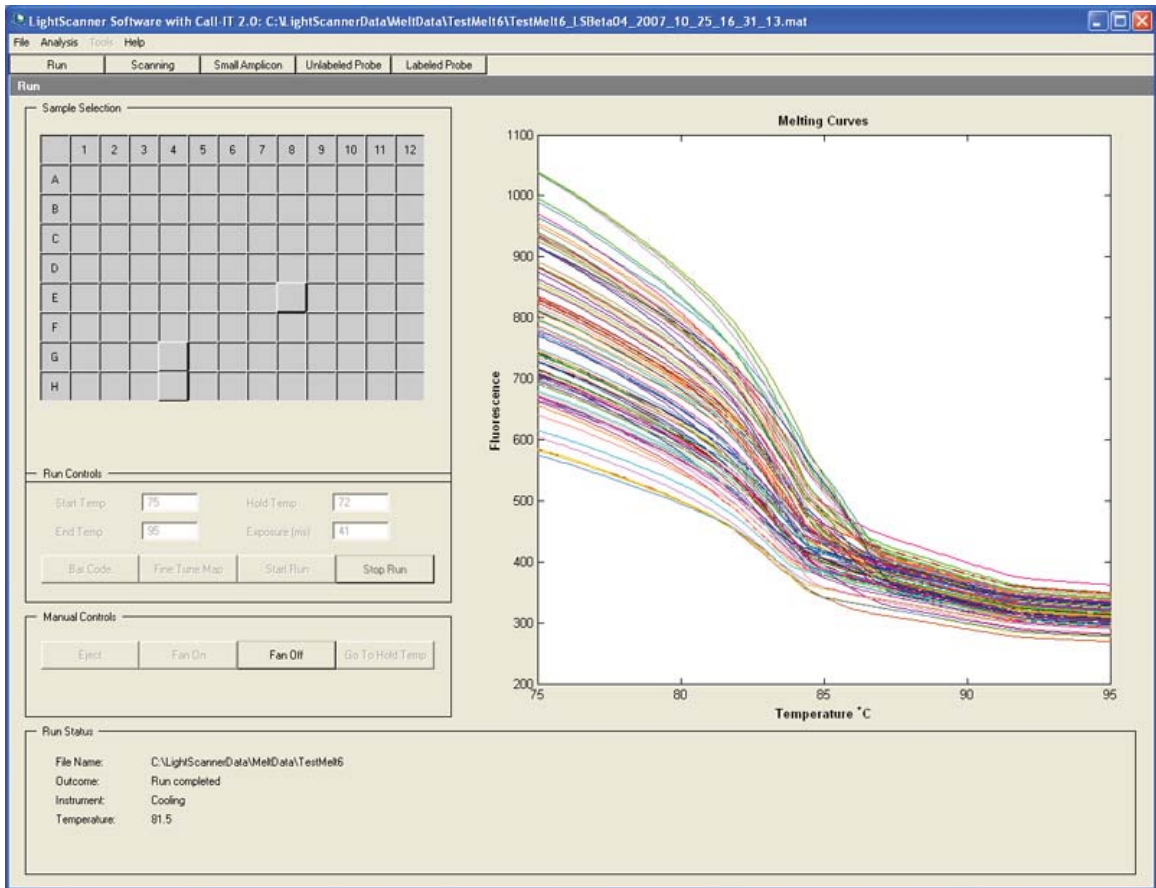
As data is collected, the software displays a chart of fluorescence versus temperature.

To View Data or Stop the Run

During the run, select the wells to be displayed using the buttons in the Sample Selection grid. If no samples are selected, the software displays the four samples in the upper left quadrant of the sample selection grid. You can display all the samples on the plate by clicking on the blank square at the upper-left corner of the grid.
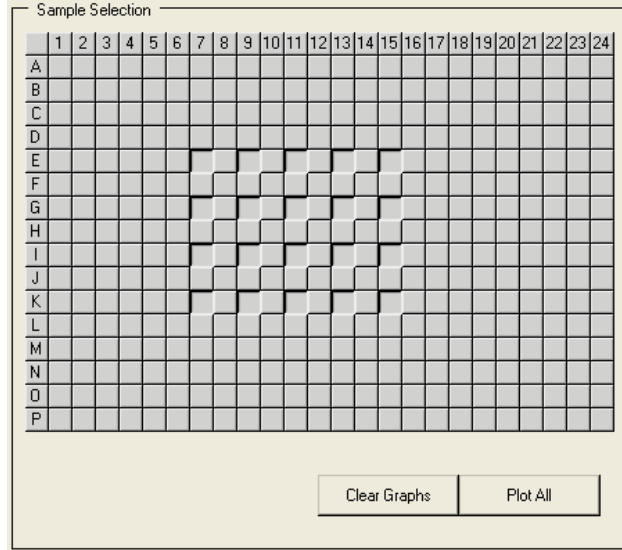
If you must end a run before the experiment is finished, click **Stop Run**. The software saves all data collected to this point. The Run Status box displays the temperature when the run was stopped.

When the experiment is finished, the instrument turns off the heat block, ejects the plate, turns on the cool-down fan and cools down to the hold temperature. The data is then loaded for analysis. The message bar displays temperature readings as the readings decrease during cool-down.

After the Run

You can access and analyze your data while the instrument is cooling down by moving directly to the Home page or by clicking one of the analysis buttons across the top.

1. To analyze the data, select either the **Scanning** or **Genotyping** button in the Analysis box.
2. To start another run, wait for the LightScanner to cool and select **New Run** from the **File** menu or run from the Home page.

CHAPTER 5

COMMON FUNCTIONS FOR ALL ANALYSES

Hi-Res Melting performs two types of data analyses: mutation scanning and genotyping. The analyses functions are accessed via the Home page.

Both types of analyses have the following common functions, each of which will be discussed in this chapter:

- Analysis subset definition
- Sample name definition
- Editing groups
- Importing analysis settings
- Working with reports
- Defining colors
- Working with graphics

Genotyping using also includes the following three options:

1. High resolution melting of very small amplicons
2. High resolution melting of unlabeled probes (LunaProbes™)
3. Melting of fluorescent probes (HybProbe® or SimpleProbe)

All three methods have advantages depending on the application. Data analysis on the LightScanner is customized to the method used, so it is important to select the correct analysis method for your application. For more information on choosing between the different genotyping methods visit **www.idahotech.com**.

The LightScanner Software Window

The LightScanner Software window consists of three primary menus to help you select and save analysis screens: the Menu Bar, Index Bar, and Analysis Subtabs.

Menu Bar: This bar contains menus to access and perform software functions.

Index Bar: This bar contains buttons used for common tasks in the current window, including subset definition, the sample editor, and the report screens. The button selections vary slightly depending on the analysis window that is currently displayed. The Index bar will display the analysis option selected from the home page, i.e., Scanning or Genotyping.

Analysis Subtabs: These subtabs are arranged sequentially to lead you through all of the steps required to complete an analysis.
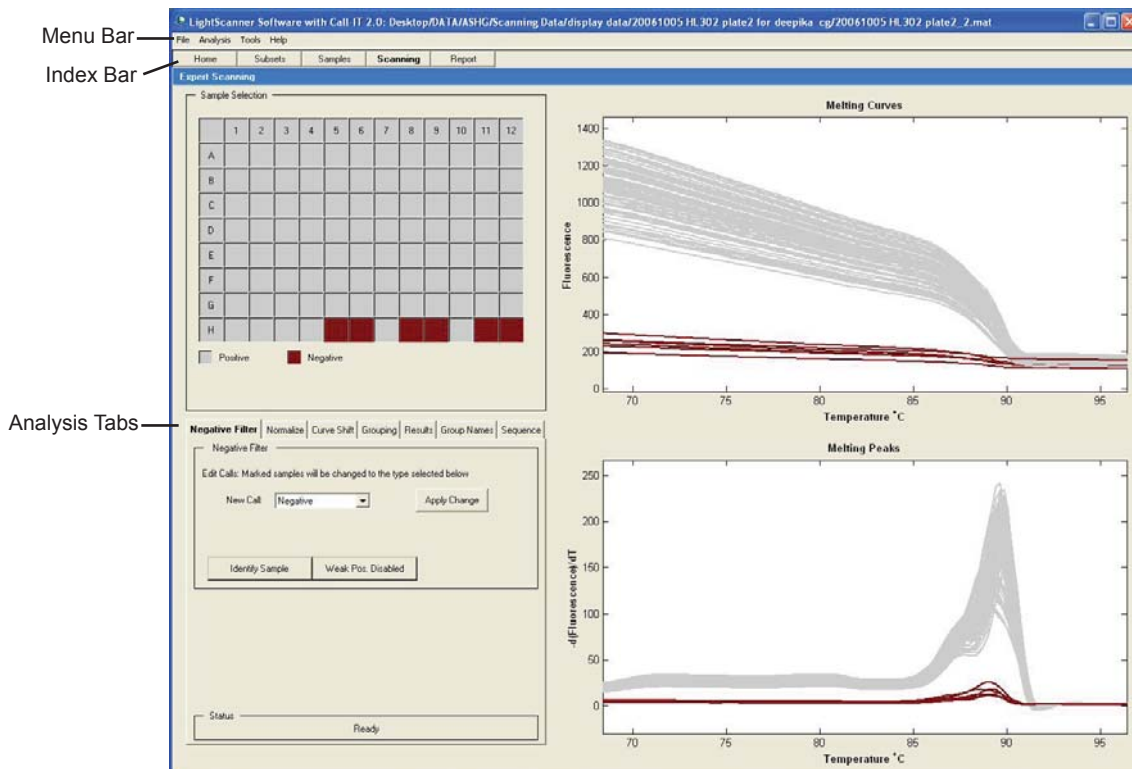
Analysis Subset Definition

Subsets can be defined by selecting the **Subsets** index tab at the top of the screen.
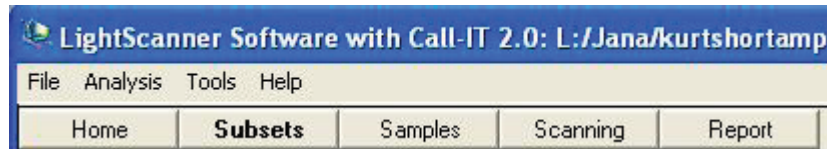
The term Subset is used to define individual amplicons that are arrayed on a single plate such that samples from a PCR fragment will be grouped and analyzed together. This is useful if you use one section of a PCR plate for one amplicon and another section of the same plate for a different amplicon. If all the samples on a plate are derived from the same amplicon, there is no need to define any subsets.

If you have divided your plate up between multiple amplicons, use the Subset screen to tell the software the locations of the samples that belong to the same amplicon set. You can define your own analysis subsets or import subset definitions that are provided with the software. The subsets provided with the software are commonly used sets, including row-wise subsets, column-wise subsets, quartered subsets, and quadrant subsets. The quadrant subsets divide 2 x 2 blocks of the plate into a checkerboard pattern of northeast, southeast, southwest, and northwest samples.

Defining a Subset

1. Select the **Subsets** index tab at the top of the screen.

2. To add a subset, select **New Subset**, then enter a subset name in the box below the Sample Selection grid. Default names are "Analysis Set 1", "Analysis Set 2" and so on.

3. To define the samples contained within a subset, click the samples in the sample selection grid or right-click and box the individual sample lines in the chart (the selected sample lines turn black).

 Note: To restore the sample colors in the chart, right-click anywhere on the chart and select **Deselect All**. To zoom in on the chart, left-click and drag across the area to be magnified. To restore the original magnification left-click anywhere on the graph.

4. Once the samples belonging to a subset have been selected, click **Add Marked to Subset** to complete the definition of the subset.

5. Repeat Steps 3 and 4 until you have defined all the subsets.

6. Select the **Export Subsets** button on the left hand side of the screen to save the subsets. The subsets will be saved as a *.sbs file.

7. Apply the saved subsets to a new data set by using the **Import Subsets** button.

To view samples in a subset, select a subset from the **Select Subset to Edit** box. In the sample grid:
- Blue boxes mark the samples in the selected subset.
- White boxes mark the samples in other subsets.
- Gray boxes mark sample locations that are not assigned to subsets.
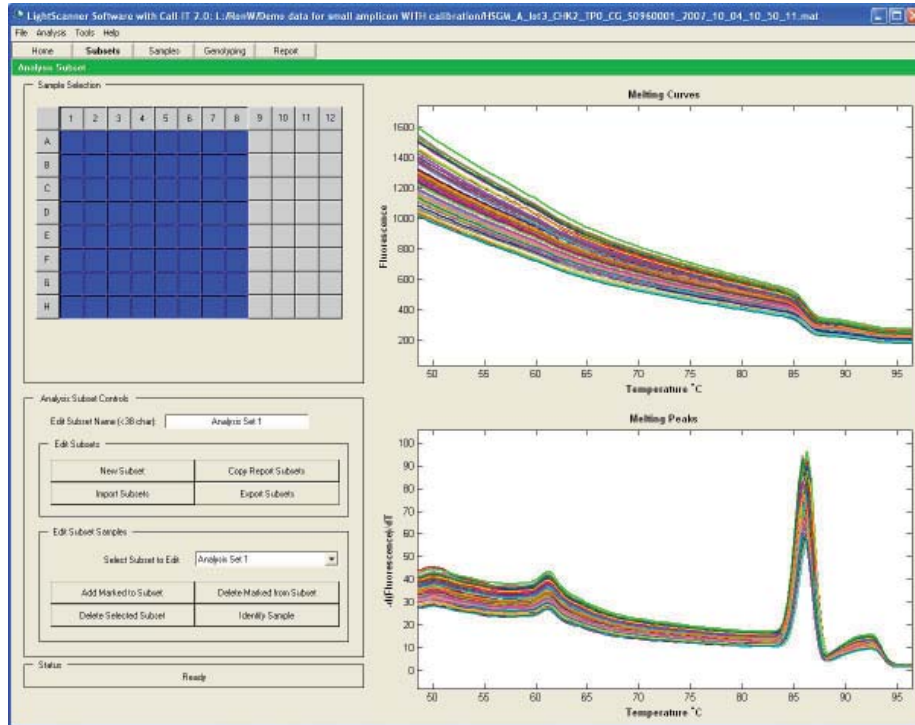
You can save time by importing frequently used subset configurations. Also, if you always lay your plate out in the same configuration you can save a custom subset configuration for re-use. The saved subset settings can then be imported into any new experiment.
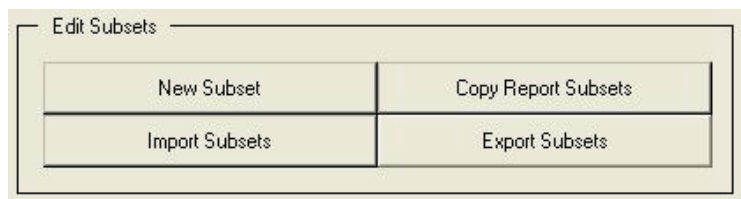
Editing a Subset

Select the subset name from the **Select Subset to Edit** box.

1. To add samples to the subset, select the samples using the sample grid or right-click on the curve in the graph, then select **Add Marked to Subset**.

2. To delete samples from the subset, select blue boxes in the grid or right-click on the curve in the graph, then select **Delete Marked** from Samples.

3. To edit the name of the subset, type the new name in the **Edit Subset Name** box.

Deleting a Subset

1. Select the subset name from the **Select Subset to Edit** box.
2. Select **Delete Subset**.
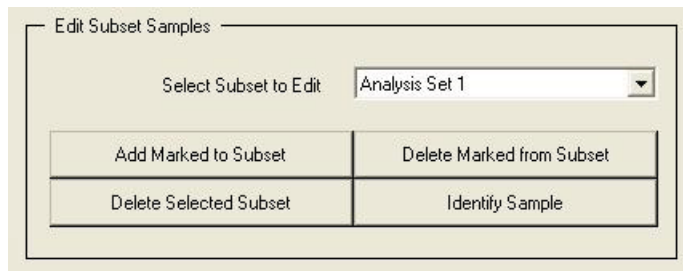
Sample Name Definition

In the LightScanner software, sample names are associated with a data set after the melting is completed. Every time an experimental file is opened, the user is able to access the Subset definition module and the Sample annotation module from the index tab at the top of the screen. Once subsets have been defined, you have the opportunity to add sample name information to your experiment. The sample editor can be accessed from the Samples tab at the top of the screen. If you have defined analysis subsets, you can display the samples by subset.
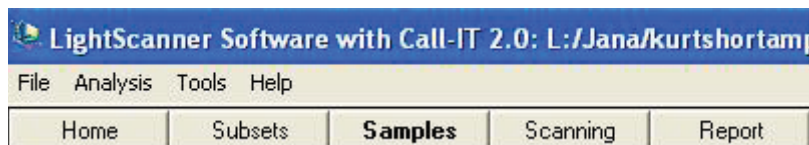
Note: Sample names can be imported in from a previously analyzed *.mat file, from a saved text file, or entered in *de novo* in the Samples tab.

Manually Entering in Sample Names for an Experiment

1. Open the Samples tab and type in the names corresponding to the sample position. If you have defined analysis subsets you can display the samples by subset.
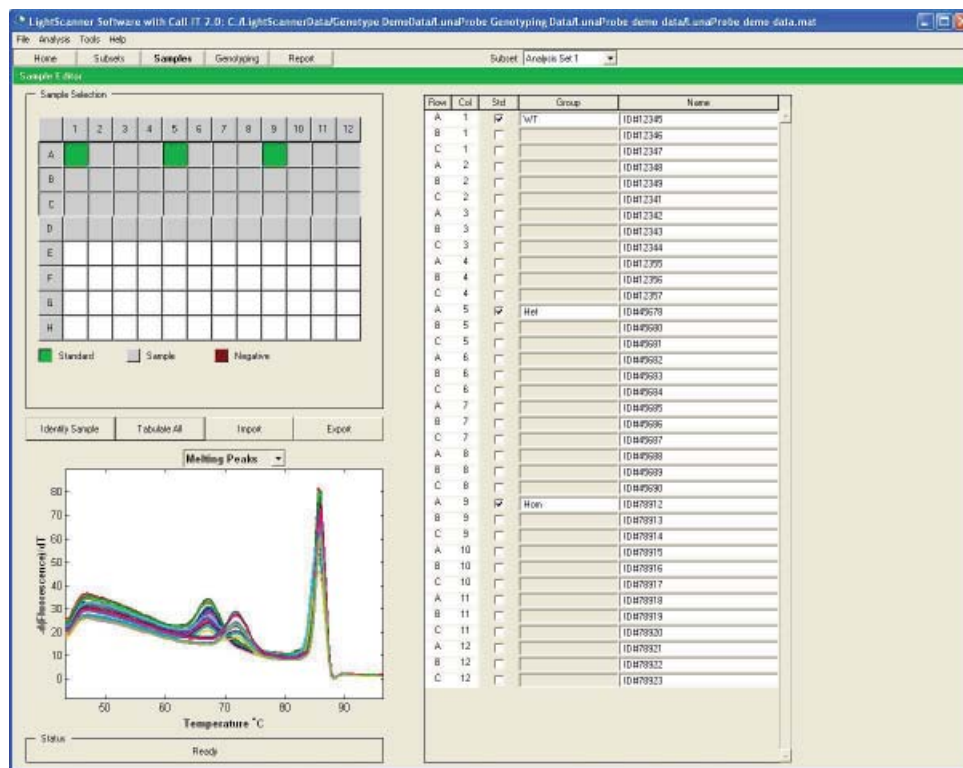
2. The sample selection grid is active in this tab and can be used to control the display. Activating a sample using the sample selection grid will display the melting data as well as the sample name in the adjacent sample editor table. Selecting the **Tabulate Selected** option will limit the table display to the samples selected in the grid, whereas choosing the **Tabulate All** option will display a list of all the sample names.

Defining a Sample as a Standard

If you are using known reference standards in your melting analysis, you can define them in the sample editor.

To select a sample as a standard:

1. Mark the check box next to the sample name in the **Std** column.

2. The Group field will now become active and you can enter the group (or cluster) name that you wish to assign to this standard (example: Wild Type).

3. The software will automatically select the **Use Internal Standards** grouping option in the subsequent analysis.

4. The assigned Group name will be used in the final grouping analysis display.

 Note: Samples assigned to the same group are expected to produce similar melting profiles to be analyzed correctly.

Duplicate samples with the same name should not be assigned different group names or a software error will result. Multiple samples that are assigned as standards with the same group name, but upon completion of the analysis are found to not fall into the same group, will result in a software error as well. If this occurs, return to the sample editor tab and de-select the outlier as a standard, and then re-analyze the edited data.

Exporting Sample Names to a *.smp File

If a common set of samples are used for several experiments you only have to enter the sample name information once and export them for use with subsequent experiments.

1. Go to the **File** menu and choose **Export** > **Export Sample Names** or select the **Export** button in the **Sample Editor** tab.

2. Name and save this exported file as a *.smp.

Importing Sample Names from a *.smp File

To associate exported sample names with a new experiment:

1. Open the new experiment

2. Go to the **File** menu and choose **Import** > **Import Sample Names** or select the **Import** button in the **Sample Editor** tab.

3. Select the file with the desired sample names. The sample name fields will automatically be populated with the imported information.

Importing Sample Names from an External Text File

Create a sample sheet template in a spreadsheet or in a tab delimited text file in the following order: Row, Column, Sample name, Standard, Group.

A	1	PID 12345	Standard	WT
B	1	PID 32792		
C	1	PID 63651		

 Note: Do not use headers in the spreadsheet. If Standards are not used in the experiment, leave the last column blank. If standards are designated in the sample sheet the software will automatically select the Use Internal Standards option as the analysis method for grouping.

Importing a sample sheet is useful when interfacing with Laboratory Information Management System (LIMS) but can also save time by allowing a user to set up a sample sheet at their own convenience, or use a standard template for multiple experiments.

Editing Groups

The final common step in all LightScanner analysis modules is the grouping of samples by melting profile. Groups are assigned a color, and all samples within a group are the same color. This section describes some of the Group editing features available in the software.

In some cases you may wish to manually overwrite the calls made by the software, either to assign an unknown sample into a particular group or to reassign a call. There are two ways to edit the grouping calls:

• You can edit an entire group by reassigning it as the Wild Type group, deleting it completely, or adding a new group into which you will manually assign samples.

• You can edit an individual sample, or a group of samples, by using the Edit Call functions.
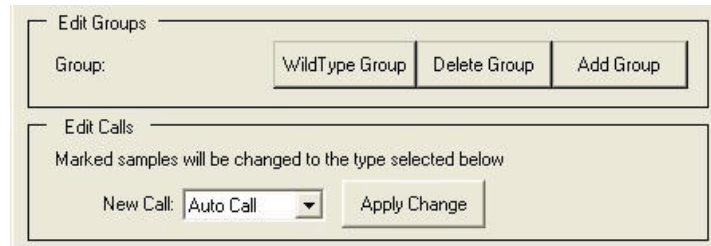
Editing a Group

1. Select the group from the pull-down menu in the **Edit Groups** field.

2. Choose to assign the group as the **Wild Type** group by selecting the button next to the group name.

3. Alternatively, delete the entire group. Note that once a group is deleted it cannot be restored without going through the analysis again.

4. Add a group. This button will add a group to the data set, giving it a letter name. This new group will not be populated with any samples until you do so by using the Edit Call functions.

Editing a Call

1. Select the sample(s) you would like to edit by clicking the sample well or right-clicking directly on the curve in the chart. You can select multiple samples by using right-click + box. Selected samples will appear as black lines on the screen.

2. In the **New Call** box, select the group that will contain the sample(s). Select **Apply Change**. The sample(s) will now be assigned to the chosen group. The sample name will be marked with an asterisk in the report table indicating that a manual change has been made to the grouping assignment.

Editing the Baseline

1. Choose the **Select Baseline** button in the grouping window.

2. The name of the button will change to **Finish Selection**.

3. Select a single sample, a set of samples, or an entire group (pick the colored box next to the group name) in the sample selection grid and select **Finish Selection**.

4. The chosen samples will be used to calculate the baseline, and the graphical display will change.

Renaming a Group

Default group names are simple one letter names. To rename a group, select the **Group Names** tab and edit the name directly in the box adjacent to the group color.
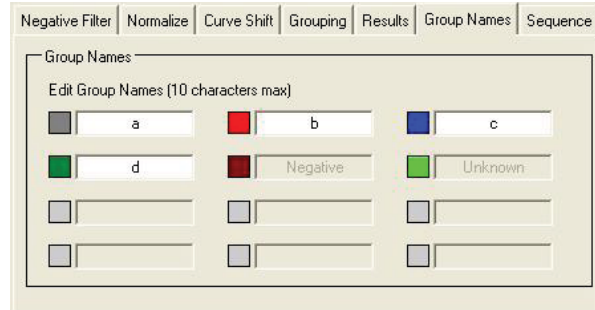
Working with Graphics

On occasion it is helpful to be able to copy the graphs and figures generated from the LightScanner software into other documents. If you have a figure that you would like to copy to the clipboard, simply right-click on the figure and select **Copy**. You can now paste the figure into a word processing program, slide program, or any other application. This feature is active for all the graphs (melting curves, melting peaks, etc.) in all the analysis modules. It is not active on the sample selection grids displayed next to the graphs.
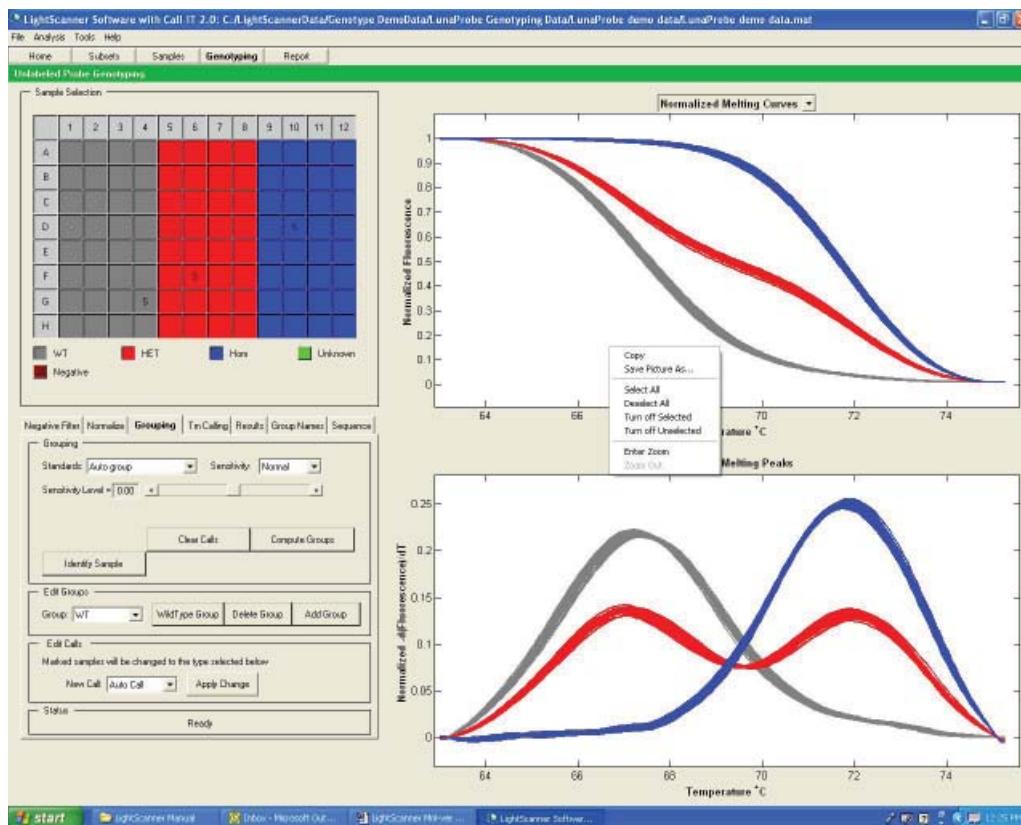
Importing Analysis Settings

It is possible to analyze a new experiment using the analysis settings from a previous experiment. In this case the subsets, the normalization cursor placements, the curve shift setting, the grouping method, and the grouping sensitivity from the reference file will all be maintained in the analysis of the new data.

The following steps are required to import analysis settings from a *.mat file:

- Open the new experiment file using the correct analysis module (Scanning or Genotyping). Analysis settings can only be applied to files that use the same analysis module (i.e., scanning file settings applied to scanning files only)

- Define any negatives in the **Negative Filter** tab.

- Go to the **File** menu and select **Import** > **Import Scanning Analysis** (or **Import Genotype Analysis**)

- You will be taken to the **Grouping** tab where the analyzed data will be displayed. At this point the analysis can be changed if required.

Note: You must remember to define the negatives before importing the analysis settings.

Working with Reports

Viewing Reports

To view a report

After you complete your analysis, click the **Reports** tab at the top of the screen. The Report Screen displays:

- A summary graph for the selected subset (if applicable) or the complete data set if no subsets were used in the analysis. If there is more than one subset, select the subset name from the Subset box in the tool bar to display the correct data.

- All of the analysis settings and parameters, captured in the display window at the upper left.

- A set of arrows at the top of the screen allow you to scroll through the detailed data reporting features. Click the buttons labeled First, Back, Next, and Last to view all the pages.

- If you have not modified the report subsets, they will be the same as the analysis subsets.

To print each page of a report, click **Print** in the upper right corner of the window.

To copy a graph, right click on the graph to bring up the menu of options for copying graphics.

Preparing Tailored Reports

A tailored report can be prepared for selected samples from a plate. If you have divided up your plate into different Analysis subsets you are not restricted to reporting the data by subset. You can prepare a tailored report (**Distinct Subset Report**) that includes samples chosen from anywhere on the plate.

For example, each column in a PCR plate might be used to analyze a different exon, while each row in the plate might represent a different patient sample. You can choose to report the data for a single row, representing the results from each exon for a single patient sample.

To prepare a tailored report

1. Move to the Report tab after completing the data analysis.
2. Select the **Subset** button at the top right of the screen.
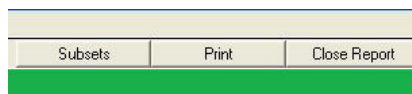
3. Create a report subset by selecting **New Subset** button, highlighting the samples you want to report together and then selecting **Add marked to Subset**.

4. As with the Analysis subsets, you can give the subset a custom name, but the default will say Report Set 1.

5. Once you define all the required Report subsets, move back to the Report window by using the button at the top of the screen.

6. Your data will now be displayed as Report subsets.

If you copy the analysis subsets into the report subsets using the Copy Analysis Subsets button, they will again remain the same as the analysis subsets.

When you define report sets, you can also specify whether the report contains the detailed sample data and the detailed sample graphs by selecting or deselecting the boxes in the Report Features section.

Defining Colors

You can customize the colors used in the LightScanner charts and displays by going to the Define Colors function.

1. From the **Tools** select **Define Colors**

2. To change the chart colors, select from the following:

Cyclic	Periodically uses 25 colors
By row	Color every row the same
By column	Color every column the same
By group	Color samples by genotype group

To define a group button color, press a group button at below the Sample Selection Grid and choose a custom color.
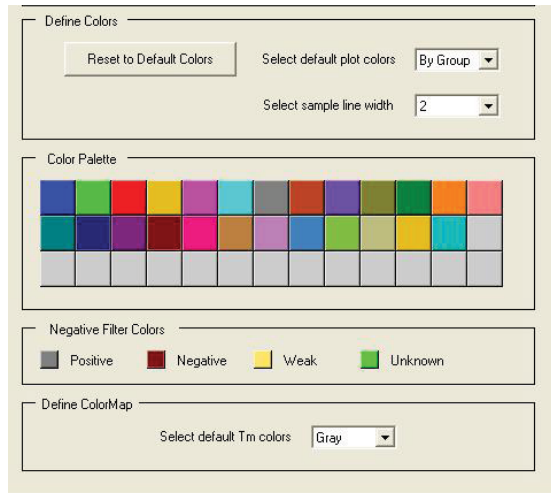
To define color maps for the Tm/Area maps, select from the options **Define Color Map** box.

CHAPTER 6

MUTATION SCANNING WITH THE LIGHTSCANNER

Introduction

Mutation scanning uses Hi-Res Melting and LCGreen Plus to detect the presence of sequence variation in a fragment of amplified DNA. The technique is especially useful in identifying heteroduplexes, which traditionally have required an additional separation step for identification. The Hi-Res Melting profile reveals heterozygous single-base changes in 2–5 min. with a sensitivity and specificity superior to nonhomogenous techniques such as denaturing high performance liquid chromatography (DHPLC) and temperature gradient capillary electrophoresis (TGCE). In addition to identifying anonymous heterozygous variants, you can also identify specific mutations. In this case, scanning and genotyping can be often combined into one simple melting analysis.

To use this analysis method after completing a run, return to the **Home** tab and select **Scanning**.

There are four steps that must be followed when analyzing Hi-Res Melting data. The steps are arranged sequentially by tabs on the software and in each case the software will present you with best-guess default settings that can be modified if necessary.

The analysis sequence is as follows:

Step 1. Negative Filter: Known negatives and failed PCR reactions should be excluded before the analysis to prevent false positive results. You can identify known negatives manually or allow the software to determine the negatives automatically

Step 2. Normalize: The melting curves are normalized to have the same beginning and ending fluorescence.

Step 3. Curve Shift: All the melting curves are brought together at a single point at the completion of denaturation to compensate for slight temperature variation across the plate and increase the accuracy of the results.

Step 4. Grouping: The software automatically clusters samples into groups with similar melting profiles.

Open a File to Analyze

1. Start the software, then select **Scanning** under the **Analysis** section of the home page.

2. Select a ***.mat** file to analyze newly collected data or inspect or re-analyze previously analyzed data, then select **Open**.

3. If you open a file that has not been previously analyzed, you will be taken directly to the Subset Selection screen. If you open an analyzed file, you will be taken to the Negative Filter selection screen.
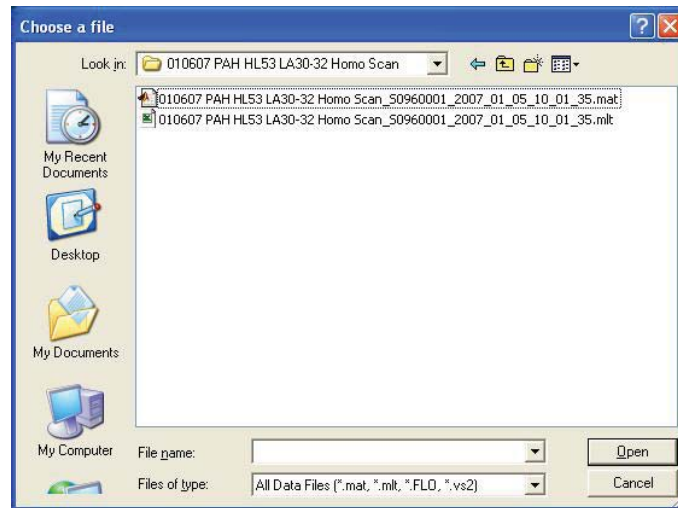
If you use the file menu to open a data set, the software will prompt you to select the appropriate analysis method.
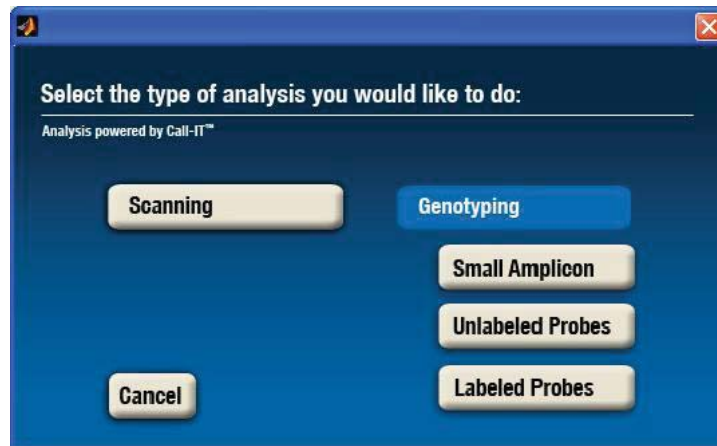
Step 1. Negative Filter

Once subsets have been defined, move to the **Scanning** tab at the top of the screen to continue the analysis. If subsets have been defined you must analyze each subset independently. A pull-down menu at the top of the screen will display the data as the selected subset. The Negative Filter is used to identify any samples that were included on the plate as negative controls and any failed PCR reactions. It is important to identify negatives before the next analysis step of data normalization to avoid the possibility of generating false positive data.

To identify negative samples manually:

1. In the **Negative Filter** tab, select **Negative** from the **New Call** pull-down menu.

2. Select samples by using right-click + drag to box the selection, or click the negative sample wells in the sample grid.

3. Select **Apply Change**.
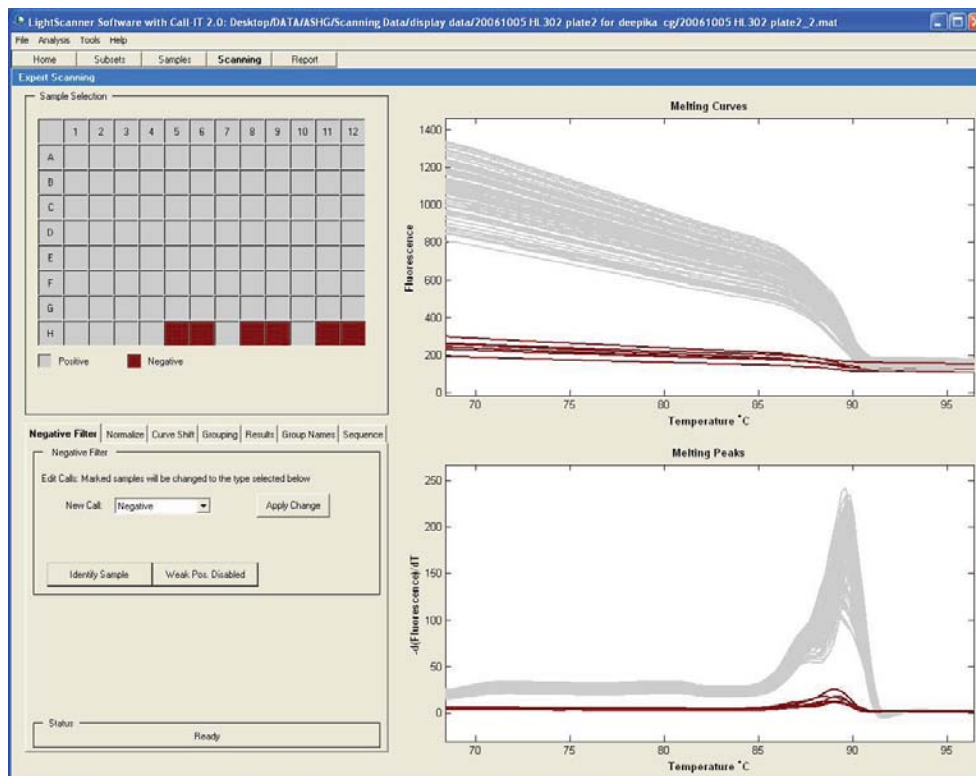
4. To auto-select negative samples, select **Auto-Call** from the **New Call** pull-down menu and select **Apply Change**.

Step 2. Normalize

Data acquired during Hi-Res Melting will vary in the magnitude of the fluorescence for each sample because of differences in the starting template or optics. Viewed this way it is often difficult to see any consistent differences between genotypes. Therefore the first step in the analysis process is to normalize the data. Two regions are selected one before and one after the major transition that will define 100% fluorescence and a 0% baseline. These regions are defined by cursors that are automatically set by the software but can be adjusted by the user if required.

To normalize the data:

1. Move to the **Normalize** tab.

2. The software automatically places the temperature cursors in a suitable region for normalization and displays the normalized data in the lower chart.

Note: The user has the option of choosing between linear or exponential background correction. Choose **Linear** (default) for single melting transitions and choose **Exponential** for complex, multi-domain melting transitions.

3. Visually confirm that the Lower Min and Lower Max temperature cursors are placed in a region of the curves, where the background fluorescence is dropping in a consistent way over all the samples, but before any temperature transitions have occurred.

4. Ideally the melting transitions should be flanked by 1–3 degrees of flat, normalized baseline regions.

5. If the cursors are located at the outer edges of the graph, pull them in towards the melting transition by dragging the cursors with your mouse or entering numerical values in the temperature boxes above the sliders.
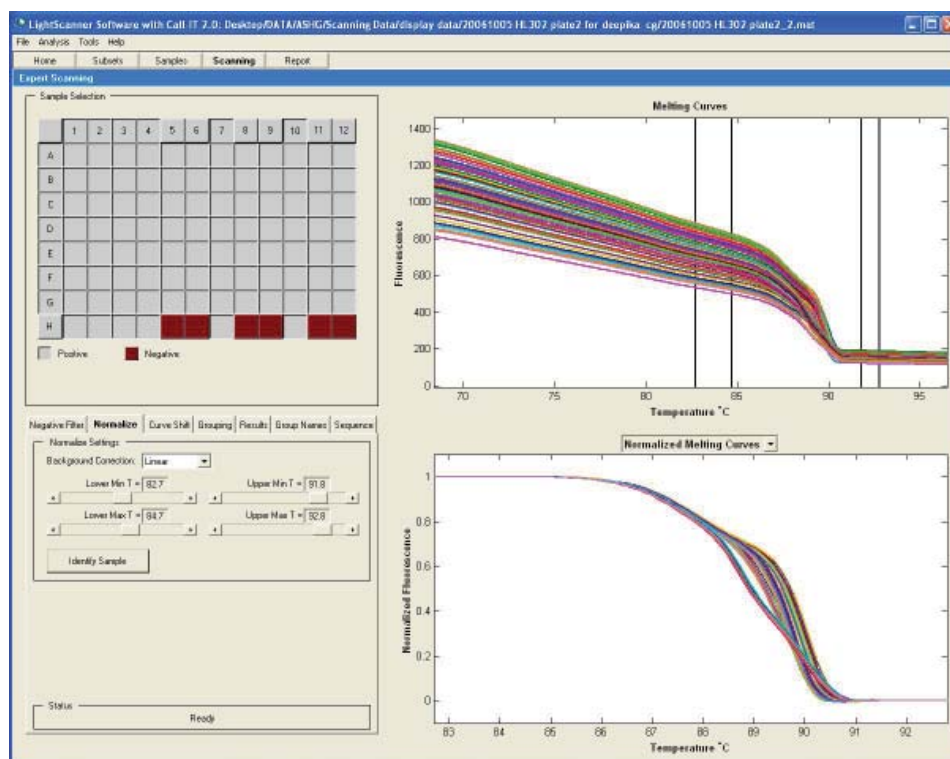
6. You can toggle between displaying normalized melting curves, temperature shifted melting curves, and difference curves using the pull-down menu in the lower graph.

Step 3. Curve Shift

The curve shift function is included in the data analysis to overcome the effect of absolute temperature variation from position-to-position across the plate. Curve shifting is performed by selecting a percent fluorescence value close to the tail end of the melting curve where all the samples are brought together. The default position chosen by the software is 5% (0.05) and is suitable for almost all applications.

By moving to the tab, the software automatically applies the Curve Shift function to the data. The upper graph shows you the original, normalized data, the lower graph shows the shifted data. In this step, samples of identical sequence should now trace identical melting profiles, and distinct clusters of profiles should become visible if variants are present in the data set.

In some cases homozygous variants can be visible more clearly before curve shifting. This is because both types of homozygous samples have the same melting profile shapes but differ by

absolute melting temperature. If this is the case with your sample set, it may be necessary to move the curve shift level. Do this by dragging the line visible on the lower graph up or down as necessary.

Be aware that eliminating the curve shift function introduces a higher level of variation to the data and may compromise the clustering of variants in the sample set, therefore it is not recommended.
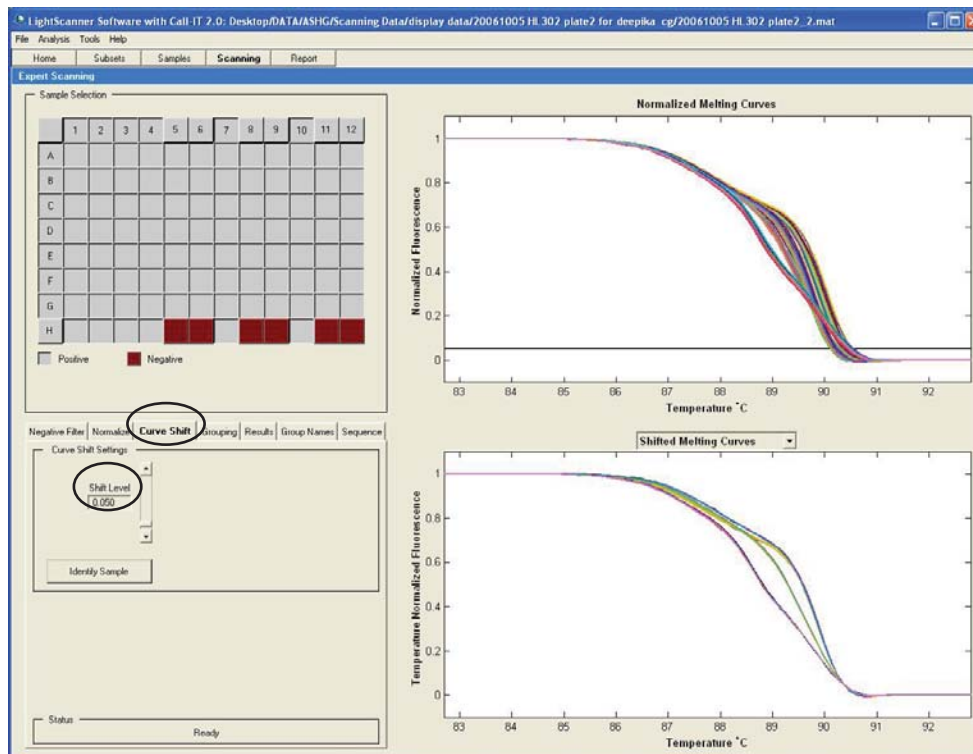
To perform curve shifting:

1. Select the **Curve Shift** tab.

2. Confirm that the normalized curves fall into distinct groups using the default setting of 0.05.

3. If the curves are not well resolved, adjust the shift level either by dragging the bar in the **Normalized Melting Curve** graph or by entering a numerical value in the **Shift Level** box. Remember the values in the box are expressed as percent maximum fluorescence.

4. You can toggle between displaying shifted melting curves and difference plots by using the pull-down menu in the lower graph. The display you choose will be carried into the next tab.

Step 4. Grouping

The final step of the analysis is to separate the samples into groups based on the shapes of the melting curves. In this tab the data is presented in two ways: a graph showing the melting curve profiles and a subtractive difference plot of the fluorescence in comparison to single a reference sample. The reference group is the one with the most numerous samples (the common group); however, the reference sample set can be changed manually by the user, if a known reference is included on the plate, by clicking on the **Select Baseline** button..
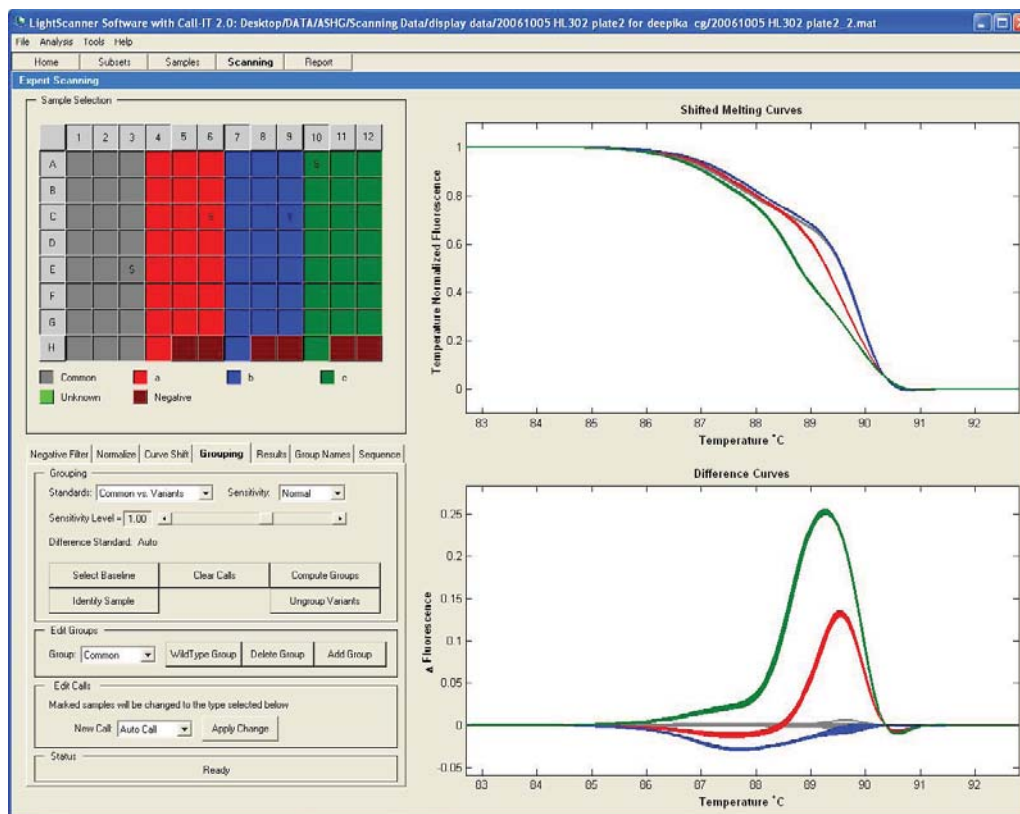
Grouping Samples Based On Curve Shape

The default grouping method is the **Common versus Variant** method. Using this method, the software selects the group with the largest number of samples and assigns it as the common group; samples with melting profiles different from the common group are put into the variant group. This method allows you to quickly select samples that are different from normal without any type of further classification. Typically these samples are then selected for sequencing to confirm the differences. If the variant group is comprised of several subgroups, select the **Group Variants** button to further classify the variants.

A second option for grouping is **Auto Group**. In this setting, samples are grouped by curve shape with no bias towards any one group. Up to 10 unique groups can be assigned to any one data set, in addition to the negative group and the unknown group which are always included despite the fact that they may not always be populated.

The third option is to group samples around known standards that are defined by the user, this method is called Use Internal Standards. The internal standards are defined by the user in the Sample Editor and automatically retrieved by the software. If duplicate standards are used, the average of the standards is used as the reference.

If the Internal Standards grouping option was selected, but only a single reference sample is used, all the samples that belong to the group containing the reference sample are grouped together. All the remaining samples are assigned as Unknown (green). To further cluster the unknown samples into individual groups, select the **Group Unknowns** button in the **Grouping** box.

1. Choose the Grouping Method

   From the **Standards** selection list, select the type of standards (Grouping Method) to be used:

   - Common versus Variant
   - Auto Group
   - Use Internal Standards

2. Choose the sensitivity—select between Normal and High:

   - Normal—select Normal sensitivity for melting profiles with single transitions.

   - High—select High sensitivity for melting profiles with two or more transitions.

   - A data set should be analyzed on a Normal sensitivity value when the standard deviation of the melt profiles of the common group is large. Conversely, when the standard deviation of the Common group is small, i.e., the data within the group are tightly clustered, you may need to use high sensitivity.

3. Adjust the sensitivity slider bar.

   Depending on the quality of the data you may need to adjust the grouping calls. The Sensitivity slider bar allows you to adjust the grouping sensitivity to a value that best fits your data. The grouping calls will change in real time as you adjust the slider, but you must press the compute groups button when you have found the desired setting to preserve the analysis.

   - The default (normal) sensitivity setting is zero.

   - To raise the number of groups, increase the value in the slider bar by moving the cursor or typing in the desired value.

   - To reduce the number of groups, decrease the value in the slider bar.

   - For the most consistent results, set the sensitivity level such that an increase or decrease of 0.3 does not change the results. This will ensure that the calculations are performed in a robust sensitivity range.

4. Select **Compute Groups** to calculate the grouping.

   Samples will be color coded by group and displayed as both Melting Curves and Difference Curves. The groupings are displayed graphically as well as on the plate map for easy cross reference. In some cases it is easier to see differences between groups on the Difference plot rather than the Melting Curve plot.

   The Difference Curve plots are computed as follows:

   a. First the software selects the group with the most number of samples.

   b. Then the standard of that group (marked with the letter S in the plate display) is used as the reference genotype. The difference in fluorescence between the reference and all other curves is plotted against Temperature.

   Note: If the Common versus Variant method is selected, the group with the most number of samples is called the common group, all other samples are defined as variants and displayed in red. To sort the variants based on curve shape, use the Group Variants button. This analysis format is illustrated in the screen capture above.

5. Save the results by going to the **File** menu and choosing **Save**. This will generate a *.mat file with the same name as the run. The *.mat file can be reopened at any time and will display all the saved analysis parameters.

View Tabulated Results

Move to the **Results** tab to view the analysis in tabulated format. Data is displayed by Row, Col, Name, and Grouping Call. If a sample has been manually reassigned to a group, it will be marked by an asterisk to flag it for future reference. These results can be exported in tab-delimited format (Excel compatible) by going to the **File** menu and choosing **Export Scanning Results**. A *.rst file will then be generated that can be opened with Excel or another spreadsheet program.

CHAPTER 7

SMALL AMPLICON GENOTYPING

Introduction

Small amplicon genotyping (SAG) is a simple yet powerful genotyping technique. No probes are required, making this technique simple, rapid, and inexpensive. Small amplicon genotyping can produce the same high accuracy results previously associated only with probe-based techniques.

How Does It Work?

High resolution melting is performed on small amplicons (50–100 bp) designed so that the primers lie immediately adjacent to the SNP of interest. For best results, choose appropriate primers using LightScanner Primer Design Software.

$$\xrightarrow{\quad\textbf{FWD}\quad} \textbf{N} \xleftarrow{\quad\textbf{REV}\quad}$$

Because the length of the amplicon is so small, all 3 species (homozygote1, homozygote 2, and heterozygote) usually have unique melting profiles that can be used to accurately genotype a sample.

Sensitivity and Specificity

The sensitivity and specificity of small amplicon genotyping have been shown to be extremely high, especially for Class I and Class II SNPs: A>C, A>G, T>C or T>G (comprising 85% of human SNPs).

Genotyping becomes more difficult when the SNP is a base neutral change (the bases switch strands but the base pair remains the same), e.g., A>T, where the homozygous AA must be differentiated from the homozygous TT. In this case the homozygous species have such subtle differences in melting profiles that experimental variation can mask the difference between the two alleles.

This limitation can be overcome by using a LightScanner High Sensitivity Genotyping Master Mix. The signal provided by temperature calibrators included in the mix is used to correct for small mechanical and chemical variations resulting in highly precise genotyping calls. In general, this Master Mix need only be used for small amplicon genotyping of polymorphisms where the melting temperature of homozygous alleles is expected to differ by less than 0.5 °C (Class III and Class IV SNPs).

The LightScanner Small Amplicon data analysis module allows data analysis of data generated with or without temperature calibrators. For simplicity, the workflow described is for experiments that include calibrators; however, only a single step is omitted when analyzing data that does not include calibrators.

Analyzing Small Amplicon Genotyping Data

Once subsets and sample names have been defined, move to the **Genotyping** index tab at the top of the screen to continue the analysis. This will put you in the negative filter screen with all the analysis sub-tabs displayed sequentially.

Step 1. Negative Filter: In this screen, known negatives and failed PCR reactions should be excluded before the analysis to prevent false positive results. You can identify negatives manually or allow the software to determine the negatives automatically.

Step 2. Calibration: In this screen, you have the option of calibrating your data to known temperature calibrators included in the High Sensitivity Genotyping master mix. The location of the calibrators is built into the software and can be selected using a pull down menu.

Step 3. Normalize: The region of the amplicon melting is bracketed by two cursors that can be adjusted by the user. Data is normalized to have the same beginning and ending fluorescence. Once the melting region is defined, the data can be viewed as melting peaks, melting curves, or difference plots.

Step 4. Grouping: The software automatically clusters samples into groups with similar melting profiles.

Move to the **Report** tab in the index bar to get a final report of your experimental analysis.

Open a File to Analyze

To open a file:

1. Start the software then select **Small Amplicon** under the **Genotyping Analysis** section of the home page.
2. Select a ***.mat** file to analyze newly collected data or to inspect previously analyzed data, then select **Open**.
3. You will be taken to the **Negative Filter** selection screen of the analysis window.
4. Alternatively, you can open a *.mlt file that contains the raw melt data, but you will need to wait a few moments as the software performs some precalculations. After this, you will be taken directly to the **Subsets** window.

Step 1. Negative Filter

Once subsets and sample names have been defined, move to the **Genotyping** index tab at the top of the screen. If subsets have been defined, you must analyze each subset independently. A pull-down menu at the top of the screen will display a list of subsets.

The Negative Filter is used to identify any samples that were included on the plate as negative controls and any failed PCR reactions. It is important to identify negatives before the next analysis step of data normalization to avoid the possibility of generating false positive data.

To identify negative samples:

1. In the Negative Filter tab, select **Negative** from the **New Call** pull-down menu.

2. Select samples by using right-click + drag to box the selection, or click the negative sample wells in the sample grid.

3. Select **Apply Change**.
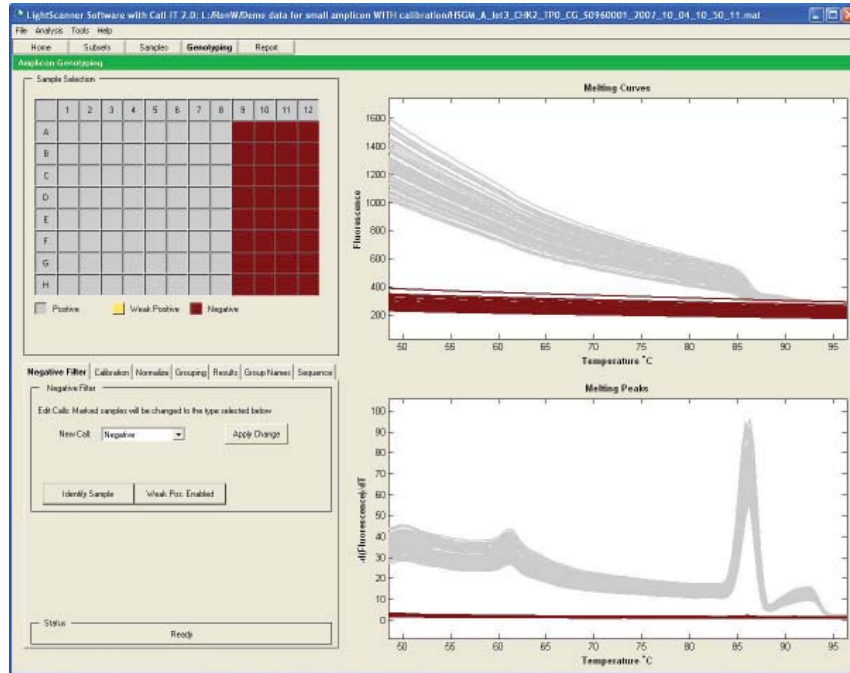
Step 2. Calibration

If you are using the High Sensitivity Genotyping Master Mix, you will need to include calibration in your analysis. The internal temperature calibrators included in the master mix are designed to melt at 60.5 ºC (Low Cal-1) and 92 ºC (High Cal-1). If you are not using calibrators in your experiment, leave the default selections (none) for the settings and move to the next tab.
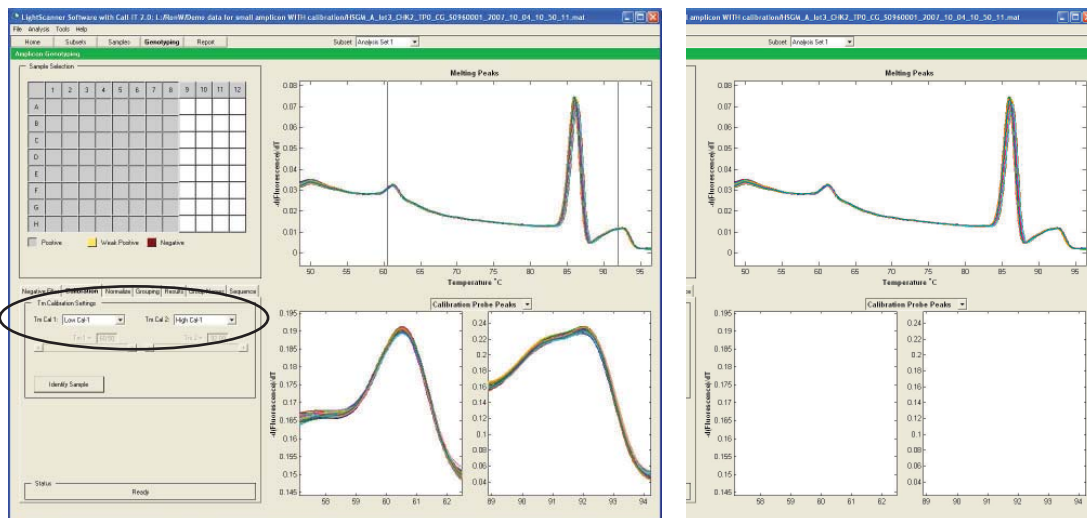
To define calibrator melting peaks:

1. Select the subset you want to analyze.

2. In the Tm Calibration Settings box, choose the positions of the Low and High Tm calibrators. If you are using the High Sensitivity master mix, choose **Low Cal-1** in the **Tm Cal-1** field, and **High Cal-1** in the **Tm Cal-2** field.

3. Cursors will automatically be placed in the regions of the calibrator melts.

Note: The cursors need only lie within the region of the melts; they do not need to lie directly on the melting peaks (see preceding figure). The software is designed to analyze each sample individually, find the correct calibrator melting peaks and make the calibration adjustments auto-matically.

Creating Custom Calibrators

If you have oligonucleotide calibrators that you have designed yourself, you have the option of either using the manual method for cursor placement, or creating a custom calibration probe library and inputting the known melting temperatures of the custom calibrators.

Manually place calibrator cursors by choosing the **Manual** method from the **Tm Calibration Settings** pull-down menu.

Create a custom calibrator probe library by going to the **File** menu and selecting **Calibration Probe Library**. A dialog box will be brought up. Enter the name of the probe and the expect-ed Tm in the box and select **Add** and **Save** to exit. This probe will now be added to the Tm Calibration Settings pull-down menu.
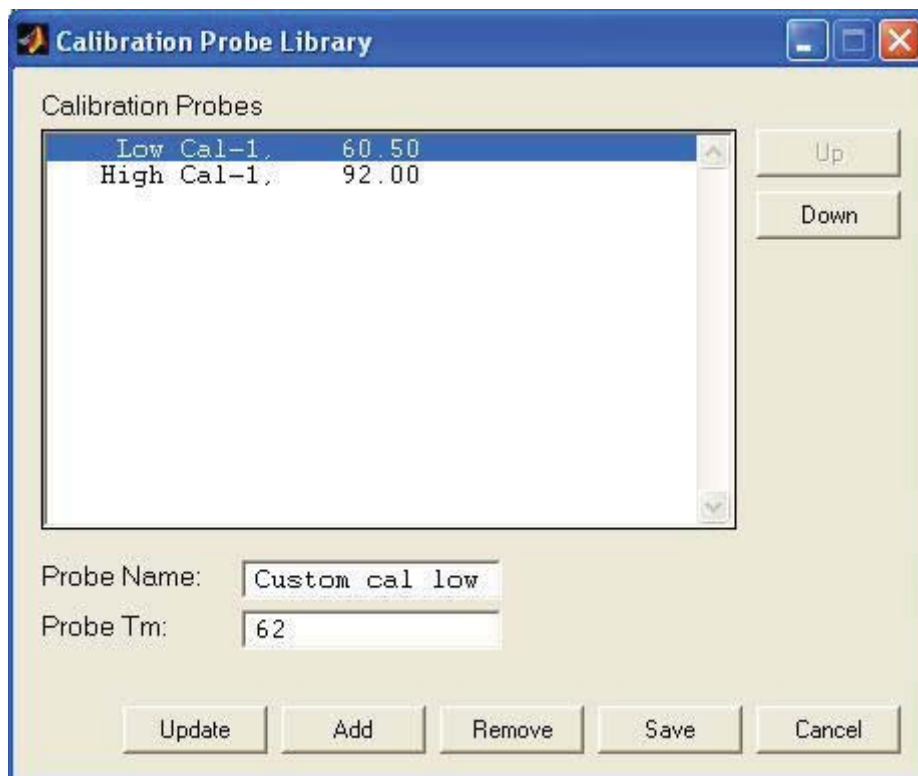
Step 3. Normalization

In this tab the primary amplicon melting peak is bracketed by two cursors. The upper graph displays the full range of fluorescence data for the run. The lower graph displays either the normalized melting curves, normalized melting peaks, or difference curves.

The software will automatically place the cursors in the assumed region of interest; however, these can be adjusted manually by dragging them with the mouse.
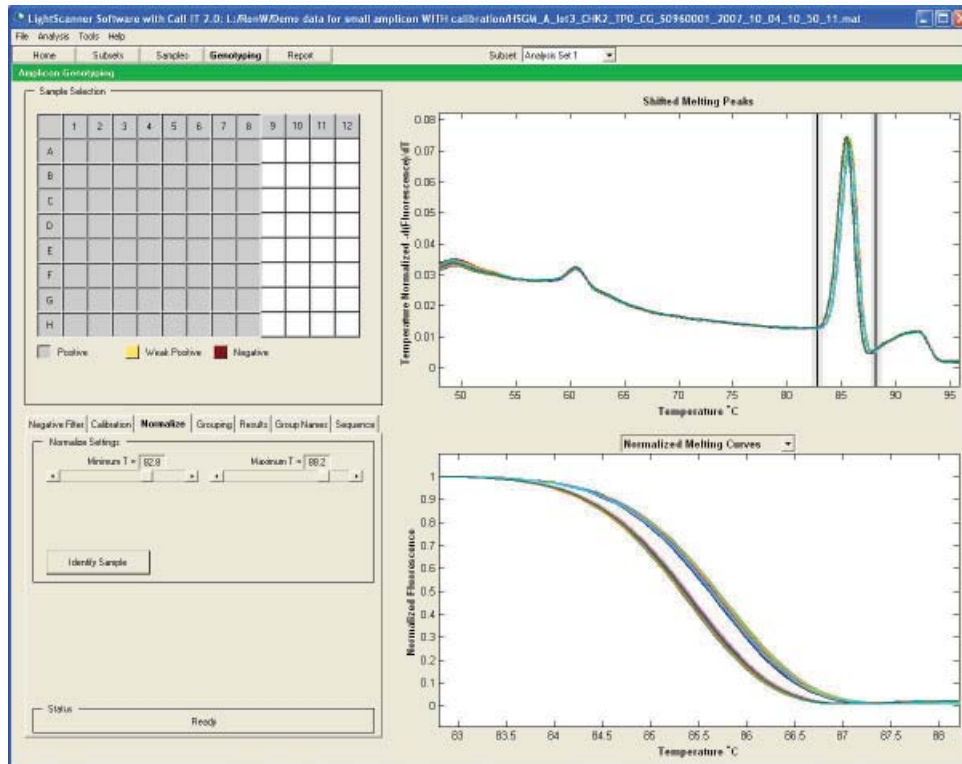
Use the melting peaks view and the melting curves view in the lower graph to confirm the correct placement of the cursors. In the shifted melting peaks view (upper graph) the cursors should be placed as close to the borders of the true peak as possible.

In the normalized melting curves view (lower graph) the beginning and ending fluorescence of all the samples should come together in a tight, flat bundle.
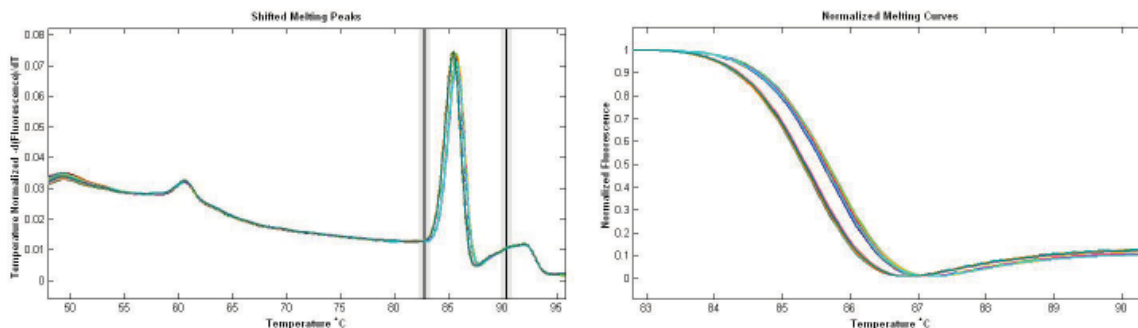
The figure above illustrates poor cursor placement resulting in an upward drift of the fluorescence at high temperatures.

Step 4. Grouping

Once the normalization step is completed, move to the **Grouping** tab to complete the analysis.

The final step of the analysis is to compute the samples into groups based on the melting pro-file of the small amplicon. In this tab, the data is presented in two graphs: the upper graph displays Normalized Melting Curves and the lower graph displays Normalized Melting Peaks, or Normalized Difference Curves.
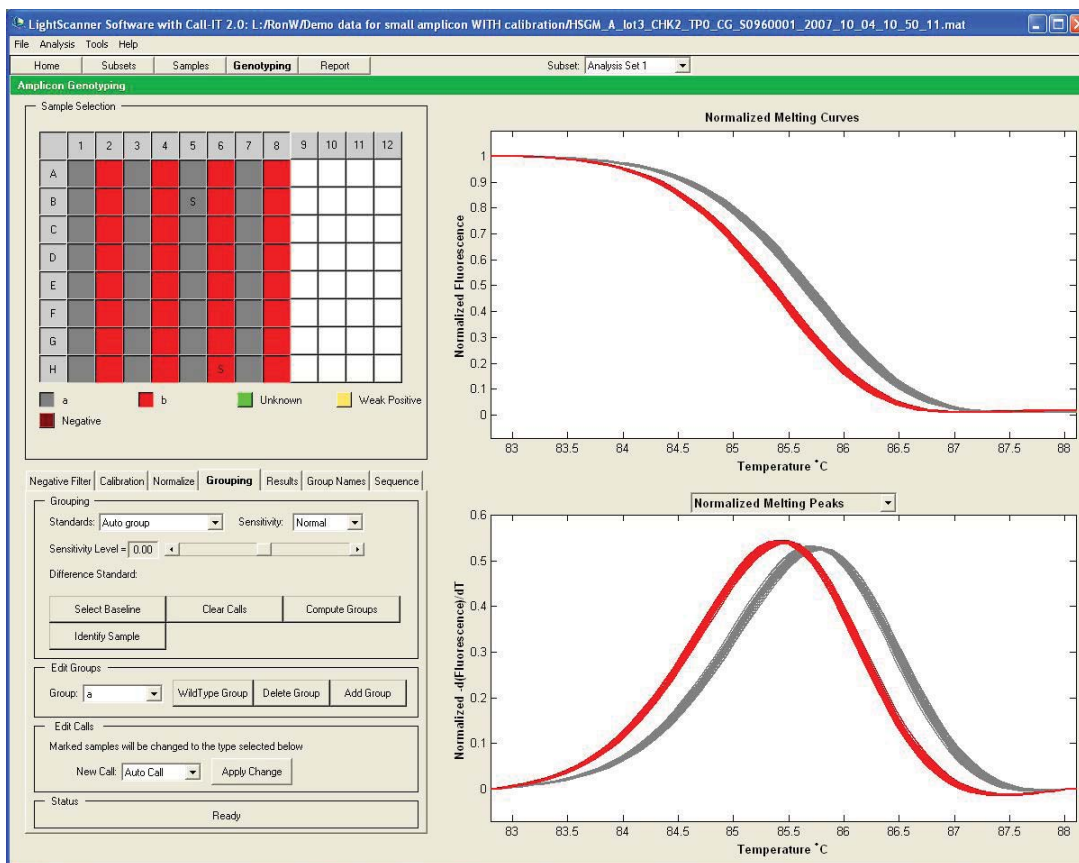

1. To compute the melting data into genotypes, first choose the grouping method from the **Standards** pull-down menu. The software offers two grouping options:

   • Auto Group—In this setting, samples are grouped by curve shape with no bias towards any one group. Up to 10 unique groups can be assigned to a data set.

   • Use Internal Standards—In this setting, samples are grouped around known standards defined by the user in the Sample Editor. If only a single reference sample is used, all the samples that belong to the group containing the reference sample are grouped together. All the remaining samples are assigned as Unknown. To further cluster the unknown samples into individual groups, select the **Group Unknowns** button in the **Settings** box.

2. Choose the sensitivity. The software offers two sensitivity settings

   • Normal—Select Normal sensitivity for peaks that are separated by 3–5 ºC.

   • High—Select High sensitivity to distinguish between groups that are separated by less than 3 ºC.

3. Adjust the Sensitivity Level. Depending on the quality of the data, you may need to adjust the grouping calls. The sensitivity slider bar allows you to adjust the grouping sensitivity to a value that best fits your data. The grouping call will change in real time as you adjust the slider, but you must select the **Compute Groups** button when you have found the desired setting to preserve that analysis. Finding the correct sensitivity is especially important when optimizing a new genotyping experiment. Once the sensitivity is established for an assay you should be able to use the same settings to analyze subsequent experiments.

    *   The default (normal) sensitivity setting is zero.

    *   To increase the number of groups, moving the slider bar to the right or type in the desired value.

    *   To reduce the number of groups, decrease the value in the slider bar.

    *   For the most consistent results between experiments, set the sensitivity level such that an increase or decrease of 0.3 does not change the results. This will ensure that the calculations are performed in a robust sensitivity range and can be used in subsequent experiments.

4. Select the **Compute Groups** button to complete the analysis.

5. Samples will be color coded by group and displayed graphically as both Normalized Melting Peaks and Normalized Difference Curves. The groupings are displayed by color both on the graphs as well as the plate map for easy cross reference.

6. Save the results by going to the **File** menu and choosing the **Save** option.

View Tabulated Results

Move to the **Results** tab to view the analysis in tabulated format. Data is displayed by Row, Col, Name, and Grouping Call. If a sample has been manually reassigned to a group, it will be marked by an asterisk to flag it for future reference. These results can be exported in tab-delimited format (Excel compatible) by going to the **File** menu and choosing **Export Scanning Results**. A *.rst file will then be generated that can be opened with Excel or another spreadsheet program.

CHAPTER 8

GENOTYPING WITH UNLABELED PROBES

Introduction

Genotyping with unlabeled is probes is achieved using the LCGreen Plus DNA binding dye in conjunction with a 3'-blocked oligonucleotide probe. In genotyping with unlabeled probes, the probe is included in the PCR mix, but is not consumed during amplification. Dissociation of the probe from the target strand is reflected in a reduction in fluorescence at the melting temperature of the probe. The key to this method is that one primer is used in excess, leading to the overproduction of the target strand. The melting transitions that are observed with unlabeled probes are quite small compared to the amplicon melts that are also observed at higher temperatures; therefore fluorescence background subtraction and normalization are critical parts of the analysis. The method provides the same specificity as current methods without the cost.

There are four steps that must be followed when analyzing Unlabeled Probe genotyping data. The steps are arranged sequentially in the analysis subtabs on the software, and at each step the software will present you with best-guess default settings that can be modified if necessary. The analysis sequence is as follows:

Step 1. Negative filter: Known negatives and failed PCR reactions should be excluded before the analysis to prevent false positive results. You can identify known false positives manually or allow the software to determine the negatives automatically

Step 2. Normalize: The melting curves are normalized to have the same beginning and ending fluorescence, and curves are displayed as derivative melting peaks.

Step 3. Grouping: The software automatically clusters samples into groups with similar melting profiles.

Step 4. Tm calling: The derivative melting peaks are used to calculate a Tm for each peak. Heterozygous samples that have two melting peaks have two Tm values displayed per sample. The difference between the two Tm values (ΔTm) is also calculated for these samples and can be used as additional analysis criteria for confirming genotypes.

Move to the **Report** tab in the **Index Bar** to get a final report of your experimental analysis.

Open a File to Analyze

To open a file:

1. Start the software then select **Unlabeled Probe** under the **Genotyping Analysis** section of the home page.

2. Select a ***.mat** file to analyze newly collected data or to inspect previously analyzed data, then select **Open**.

3. You will be taken to the **Negative Filter** selection screen of the analysis window.

4. Alternatively, you can open a ***.mlt** file that contains the raw melt data, but you will need to wait a few moments as the software performs some precalculations. After this, you will be taken directly to the **Subsets** window.

Step 1. Negative Filter

The Negative Filter is used to identify any samples that were included on the plate as negative controls and any failed PCR reactions. It is important to identify negatives before the next analysis step of data normalization to avoid the possibility of generating false positive data. If subsets have been defined, you must analyze each subset independently. A pull-down menu at the top of the screen will display the data is the selected subset.

To identify negative samples:

1. In the **Negative Filter** tab, select **Negative** from the **New Call** pull-down menu.

2. Select samples by using right-click + drag to box the selection, or click the appropriate sample wells in the sample grid.

3. Select **Apply Change**.
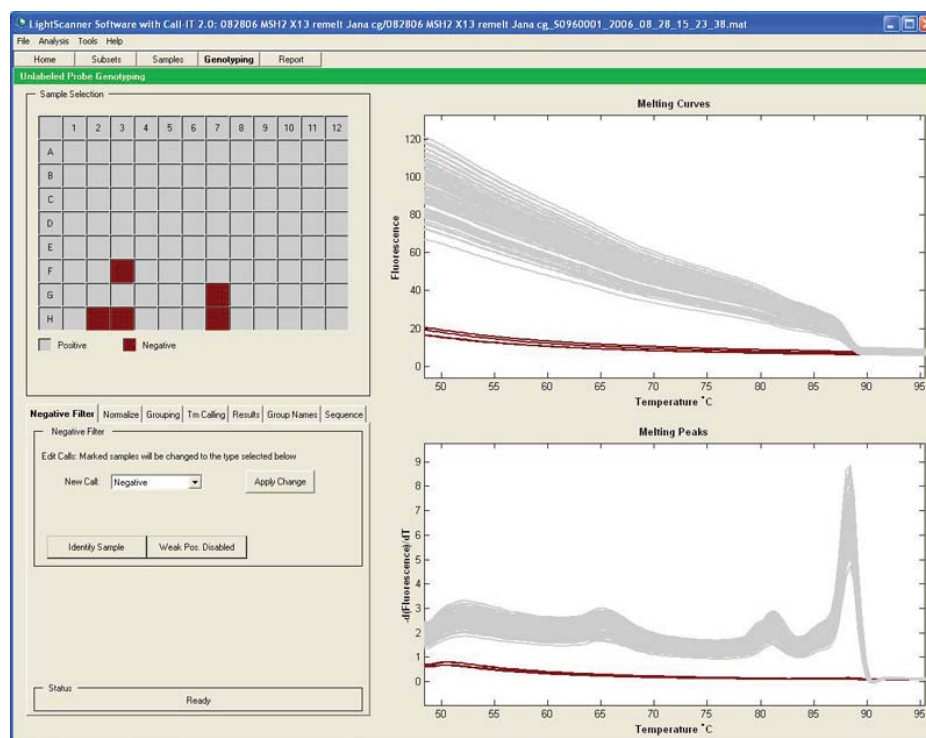
Step 2. Normalize the Data

To normalize the data for unlabeled probes:

1. Move to the **Normalize** tab.

2. The upper chart displays the full range of fluorescent data for the run. The software automatically performs the background subtraction and places the temperature cursors in a suitable region for normalization. Visually confirm that the Minimum and Maximum temperature cursors bracket the region of the probe melt.
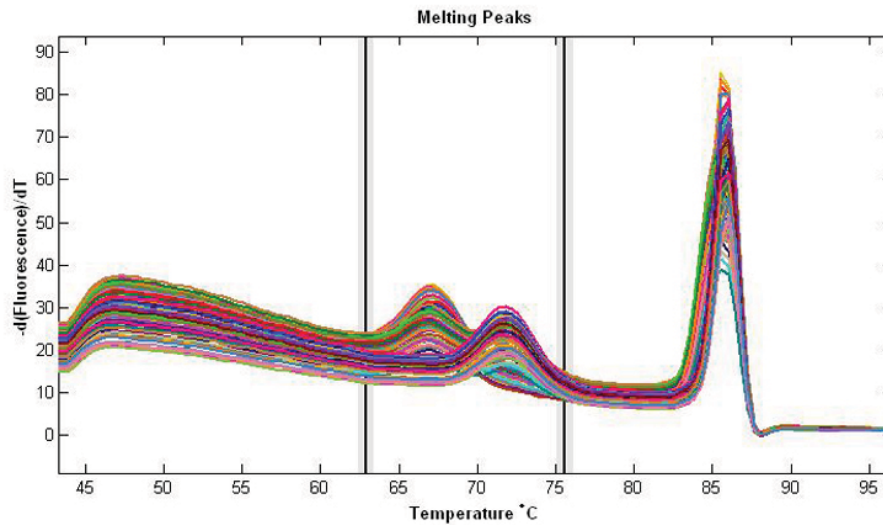
3. The lower chart displays the normalized derivative melting peaks of the data.
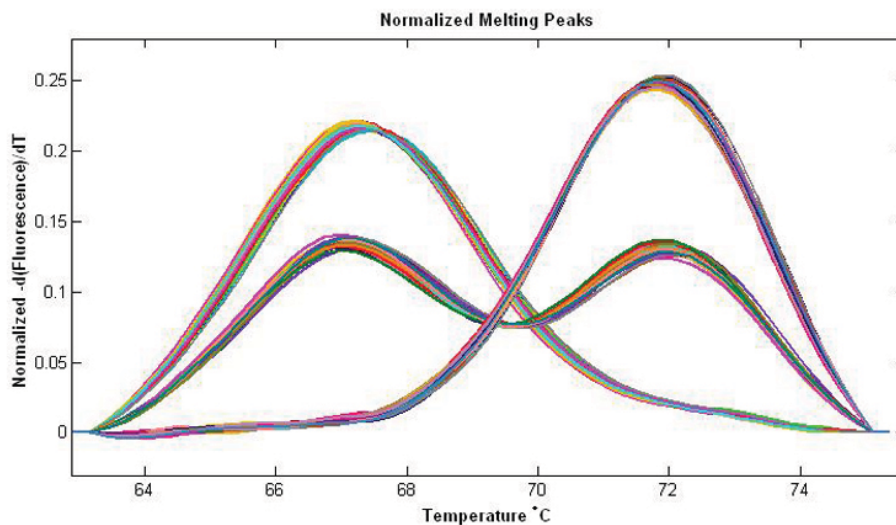
4. Toggle the lower graph between melting peaks and melting curves to confirm that the cursor placement is correct. In the melting curves graph, you should observe a completely flat region before and after the melting transition to get the best results.

Normalized Melting Curves
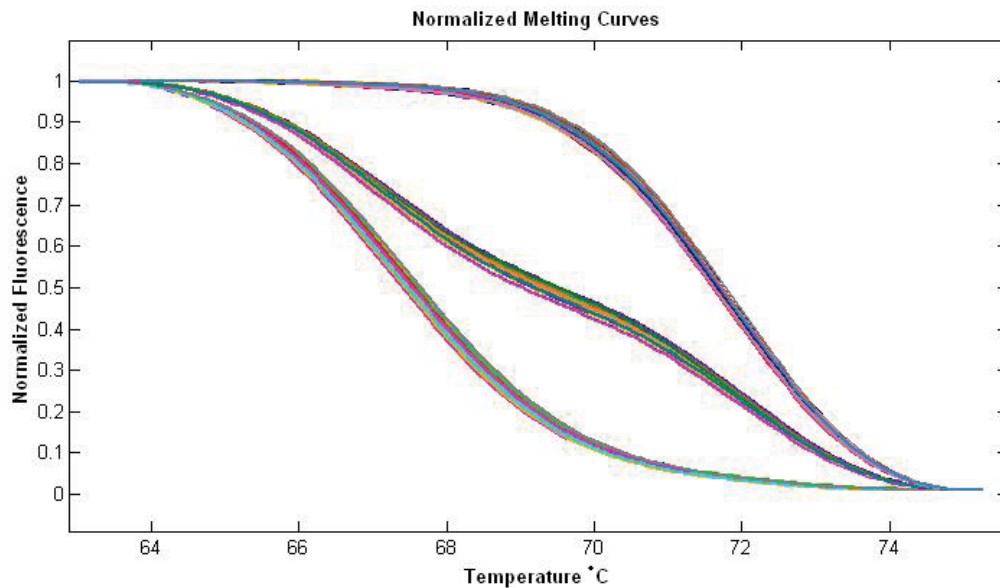

5. If the cursors are located at the outer edges of the graph, pull them in towards the melting transition by dragging the cursors with your mouse or entering numerical values in the temperature boxes above the sliders.

Step 3. Grouping

The next step of the analysis is to compute the samples into groups based on the melting profile of the probe. In the Grouping tab, the data is presented in two graphs. The upper graph is directly carried over from the previous screen and can be toggled between melting curves and melting peaks. The lower graph displays melting peaks only. The upper graph always displays all the samples in the Analysis. The lower graph displays user selected samples.
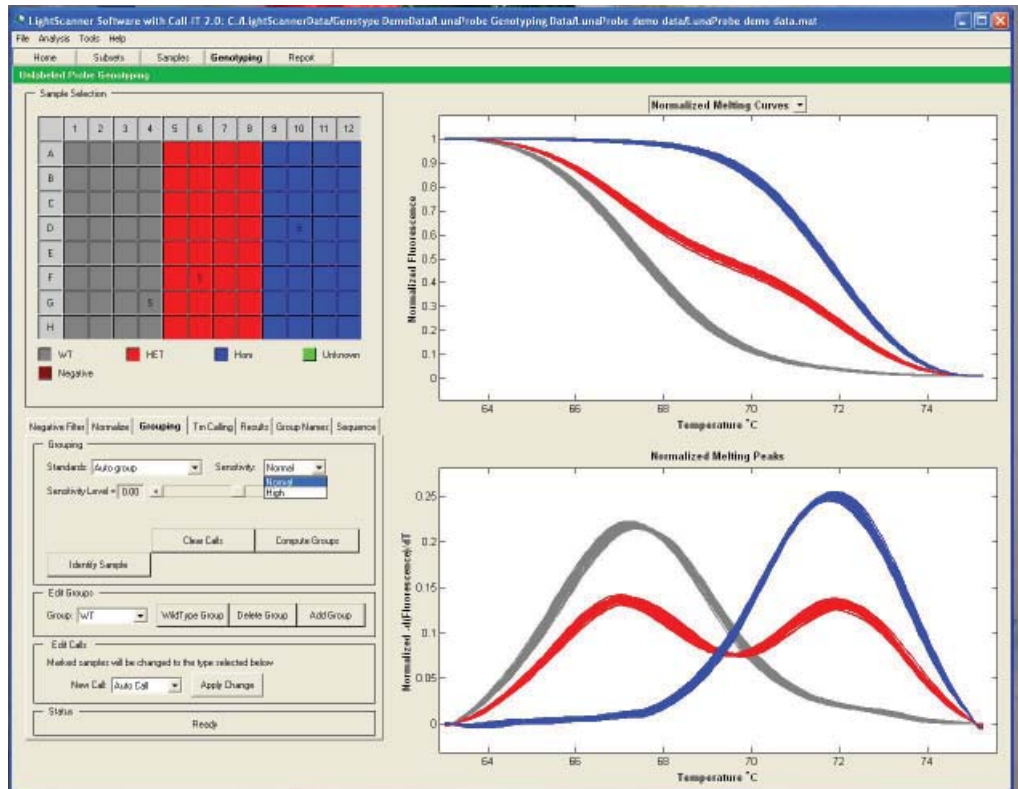
1. To compute the melting data into genotypes, first choose the grouping method from the **Standards** pull-down menu. The software offers two grouping options:

    •   Auto Group—In this setting, samples are grouped by melting temperature with no bias towards any one group. Up to 10 unique groups can be assigned to a data set.

    •   Use Internal Standards—In this setting, samples are grouped around known standards defined by the user in the sample editor. If only a single reference sample is used, all of the samples that belong to the group containing the reference sample are grouped together. All the remaining samples are assigned as Unknown. To further cluster the unknown samples into individual groups, select **Group Unknowns**.
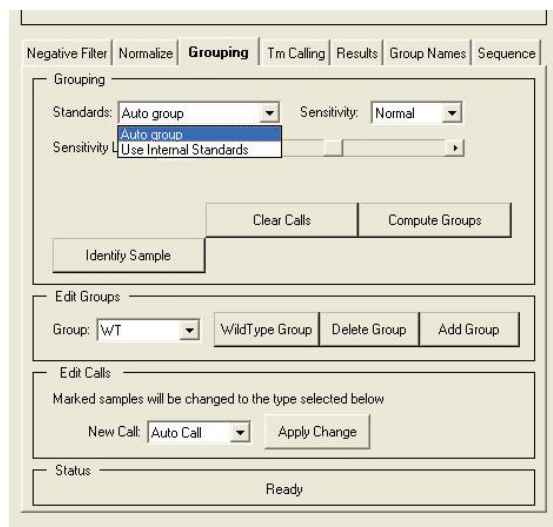
2. Choose the sensitivity. The software offers two sensitivity settings

   • Normal—Select Normal sensitivity for peaks that are separated by 3–5 ºC.

   • High—Select High sensitivity to distinguish between groups that are separated by less than 3 ºC.
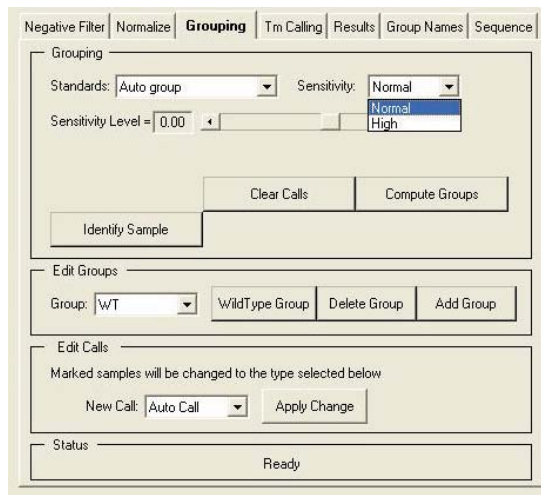
In general, normal sensitivity should be used for peaks that are separated by 3–5 ºC. The High sensitivity setting may be needed for peaks that are less well separated (see example below)
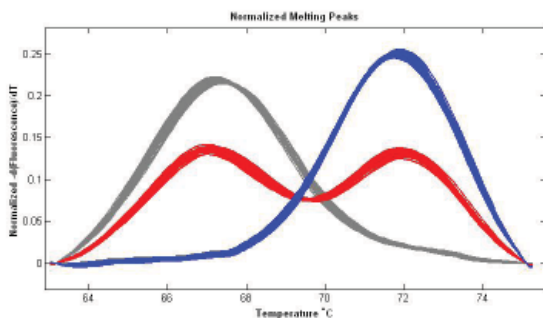
Normal Sensitivity Example

High Sensitivity Example

3. Adjust the sensitivity level.

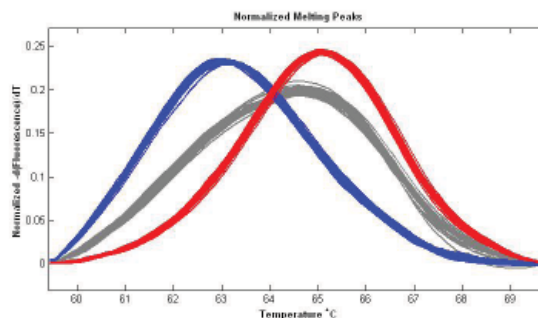   Depending on the quality of the data, you may need to adjust the grouping calls. The sensitivity slider bar allows you to adjust the grouping sensitivity to a value that best fits your data. The grouping call will change in real time as you adjust the slider, but you must select the **Compute Groups** button when you have found the desired setting to preserve that analysis. Finding the correct sensitivity is especially important when optimizing a new genotyping experiment. Once the sensitivity is established for an assay, you should be able to use the same settings to analyze subsequent experiments.

   • The default (normal) sensitivity setting is zero.

   • To increase the number of groups, move the slider bar to the right or type in the desired value.

   • To reduce the number of groups, decrease the value in the slider bar.

- • For the most consistent results between experiments, set the sensitivity level such that an increase or decrease of 0.3 does not change the results. This will ensure that the calculations are performed in a robust sensitivity range and can be used in subsequent experiments.

4. Select the **Compute Groups** button to complete the analysis.

5. Samples will be color coded by group and displayed graphically as both Normalized Melting Peaks and Normalized Difference Curves. The groupings are displayed by color both on the graphs as well as the plate map for easy cross reference.

6. If the Internal Standards grouping option was selected, but only a single reference sample is used, all the samples that belong to the group containing the reference sample are grouped together. All the remaining samples are assigned as Unknown (green). To further cluster the unknown samples into individual groups select the **Group Unknowns** button in the **Grouping** box.

7. Save the results by going to the **File** menu and choosing **Save**.

Step 4. Tm Calling

The melting temperature (Tm) of a sample is defined as the point in a melting curve where 50% of the probe-target hybrid is dissociated. The LightScanner software is designed to automatically calculate the melting temperature for every sample.

To calculate the Tm automatically, select the **Automatic Mode** method, then do the following:

1. In the **Selection** box pull-down menu, select **Genotype**. This option groups samples by the genotyping assignments made in the previous screen.

2. Select **Normal** in the **Sensitivity** box if you only want to analyze the major peaks. The Tm may appear to be skewed due to contributions from minor peaks. Select **High** in the **Sensitivity** box if you want to analyze major and minor peaks independently.

3. Finally, select **Compute Tm Values**.

4. The results will be displayed in tabulated form in the Tm Calling Data display below the graph. For heterozygous samples, an additional calculation of ΔTm is also displayed.

5. The data table will display the following data:

    Tm1   The melting temperature of the first peak in the sample
    Tm2   The melting temperature of the second peak in the sample
    ΔTm   The difference in the melting temperatures between the 2 peaks

6. Data that the software automatically calculates but does not display includes peak area, height, and width and shoulder regions. This information can be displayed by selecting the check boxes in the Data Display frame.

Optional data	
Area 1	The area of the first peak in the sample
Area2	The width of the first peak in the sample
Width1	The area of the second peak in the sample
Width2	The width of the second peak in the sample
Height1	The height of the first peak in the sample
Height2	The height of the second peak in the sample

7. Data can be sorted by Group, Row, Column, Name, or Tm by clicking on the column headers.

8. Genotyping calls can be edited based on Tm assessment by using the Edit Calls menu. Right click + box the samples you wish to edit. Use the options in the pull down menu to select the new call, and select **Apply Change**.

9. A summary of all the results is compiled in the Results tab window. These results can be exported as a tab delimited file for data management purposes. To export the data, go to **File>Export>Genotyping Results** under the Menu Bar.

Manual Tm Calling

You can also determine peak area and Tm manually. Manual Tm should be calculated ***one sample*** at a time.

1. Select a single sample from the sample grid.

2. Under **Method** choose **Manual** mode. For Labeled Probe Analysis, the Tm Calling Editor below the graph now displays two check boxes if the peaks option is set at two peaks or less, and six check boxes if six peaks or less is selected. For Unlabeled Probe Analysis, two check boxes are displayed.

3. Checking the boxes immediately brings up a cursor on the screen that can be dragged to any position on the graph. The position of the cursor is indicated numerically next to the check box. This is the manually calculated Tm.

4. Tm values are computed in real-time, and the compute Tm values button is inactive.

Directed Auto Mode for Tm Calling

The Directed Auto Mode is designed for situations where the user knows the expected melting temperature of the peaks they are interested in. The Tm cursor is placed in the expected region of probe melting, and the software limits the Tm calculation to the region indicated. This method can be useful for samples where low allele fractions (or low concentrations of sample) give small melting peaks that are not recognized by the Automatic Tm calling algorithms. In this case, the software will search the region surrounding the Tm cursor, look for anything that is considered a melting peak, and assign a Tm.

The Directed Auto Mode should only be used with a single sample or a cluster of samples with melting peaks in the same Tm range. If the method is used with samples that have very different melting temperatures, the software will place the cursors in locations suitable for sample A1 and calculate and display the Tm based on these regions only.

1. Select a single sample or a cluster of samples with similar Tm for analysis.

2. Select **Genotype** or **Peak Number** in the **Selection** box.

3. Select Directed Auto Mode from the Method pull down menu. This will bring up two check boxes below the Melting Peaks graph. Checking a box will bring up a cursor on the screen that can be dragged to the region around which the software should perform the peak search.

4. Tm values are calculated in real-time, and the Compute Tm Values button is inactive.

5. Tm values are displayed in the Tm Calling data table. Note that in contrast to the Manual Tm calling mode, each sample will have a slightly different Tm.

6. Save the results by going to the **File** menu and choosing **Save**. Results can also be exported by choosing **Export Genotyping Results** from the **File** menu.

View Tabulated Results

Move to the **Results** tab to view the analysis in tabulated format. Data is displayed by Row, Col, Name, and Grouping Call. If a sample has been manually reassigned to a group, it will be marked by an asterisk to flag it for future reference. These results can be exported in tab-delimited format (Excel compatible) by going to the **File** menu and choosing **Export Scanning Results**. A *.rst file will then be generated that can be opened with Excel or another spreadsheet program.

CHAPTER 9

GENOTYPING WITH LABELED PROBES

Introduction

Genotyping with labeled is probes is achieved using either HybProbes or SimpleProbes. Fluorescence resonance energy between the dyes on adjacent HybProbes generates a signal that decreases as the temperature is increased. With SimpleProbes, the fluorescence is high when the dye-conjugated probe is bound to the target strand and is lost as the duplex melts. The melting transitions that are observed with labeled probes are large, and not confounded by the presence of the amplicon melting region, therefore it is not necessary to bracket the melt regions as carefully as required with the unlabeled probe analysis.

In the Labeled Probe application, after background subtraction, melting transitions are displayed as normalized melting curves and melting peaks representing the negative derivative of fluorescence versus temperature (-dF/dT). The Tm values calculated from the melting peaks can then be used to characterize genotypes.

There are four steps that must be followed when analyzing Labeled Probe genotyping data. The steps are arranged sequentially in the analysis subtabs on the software, and at each step the software will present you with best-guess default settings that can be modified if necessary.

The analysis sequence is as follows:

Step 1. Negative filter: Known negatives and failed PCR reactions should be excluded before the analysis to prevent false positive results. You can identify known false positives manually or allow the software to determine the negatives automatically

Step 2. Normalize: The melting curves are normalized to have the same beginning and ending fluorescence and curves are displayed as derivative melting peaks.

Step 3. Grouping: The software automatically clusters samples into groups with similar melting profiles.

Step 4. Tm Calling: The derivative melting peaks are used to calculate a Tm for each peak. Heterozygous samples that have two melting peaks have two Tm values displayed per sample. The difference between the two Tm values (ΔTm) is also calculated for these samples and can be used as additional analysis criteria for confirming genotypes.

Move to the **Report Tab** in the **Index Bar** to get a final report of your experimental analysis.

Open a File to Analyze

To open a file:

1. Start the software then select **Labeled Probe** under the **Genotyping Analysis** section of the home page.

2. Select a ***.mat** file to analyze newly collected data or to inspect previously analyzed data, then select **Open**.

3. You will be taken to the **Negative Filter** selection screen of the **Analysis** window.

4. Alternatively, you can open a *.mlt file that contains the raw melt data, but you will need to wait a few moments as the software performs some precalculations. After this, you will be taken directly to the **Subsets** window.

Step 1. Negative Filter

The Negative Filter is used to identify any samples that were included on the plate as negative controls and any failed PCR reactions. It is important to identify negatives before the next analysis step of data normalization to avoid the possibility of generating false positive data. If subsets have been defined, you must analyze each subset independently. A pull-down menu at the top of the screen will display the data in the selected subset.

To identify negative samples:

1. In the **Negative Filter** tab, select **Negative** from the **New Call** pull-down menu.

2. Select samples by using right-click + drag to box the selection, or click the appropriate sample wells in the sample grid.

3. Select **Apply Change**.
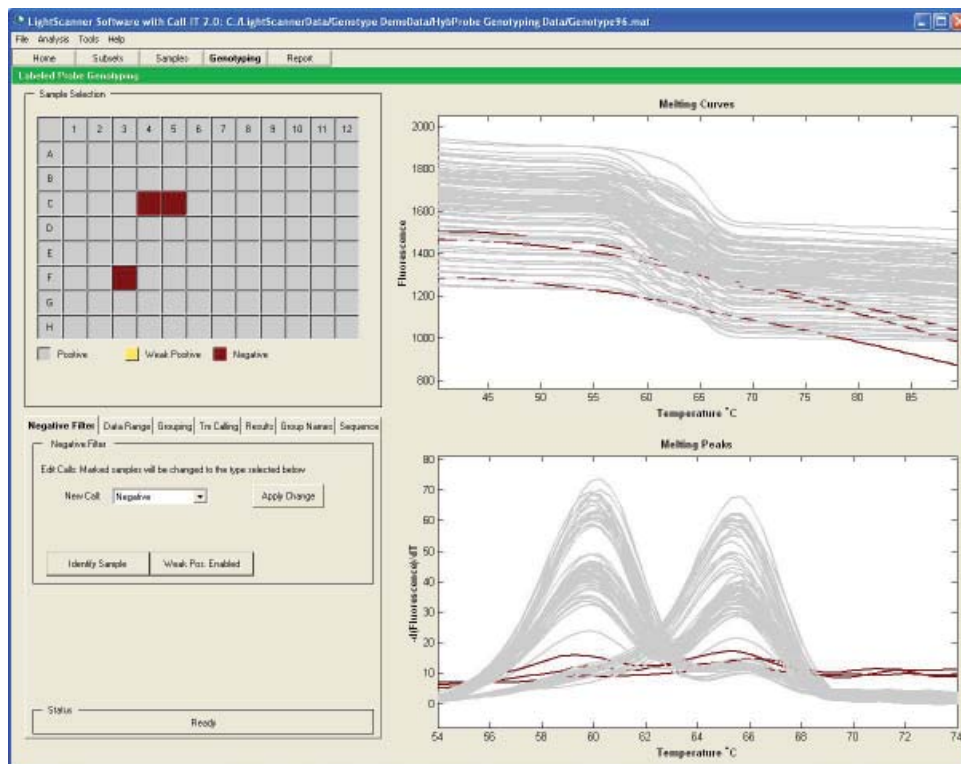
Step 2. Data Range

To set the data range for labeled probes:

1. Move to the **Data Range** tab.

2. The upper chart displays the full range of fluorescent data for the run. The software automatically performs the background subtraction and places the temperature cursors in a suitable region for analysis. Visually confirm that the Minimum and Maximum temperature cursors bracket the region of the probe melt.
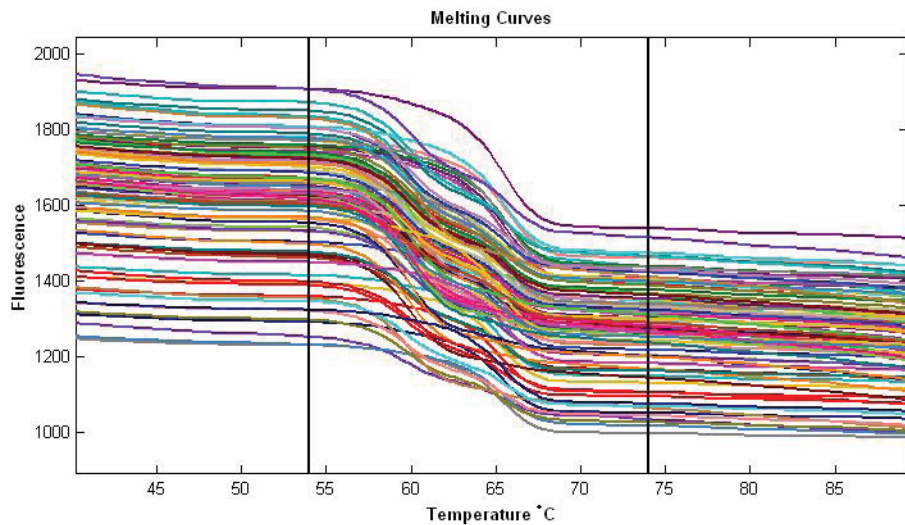
3. The lower chart displays the background corrected derivative melting peaks of the data (-dF/dT versus Temperature) with an option to display raw melting curves.
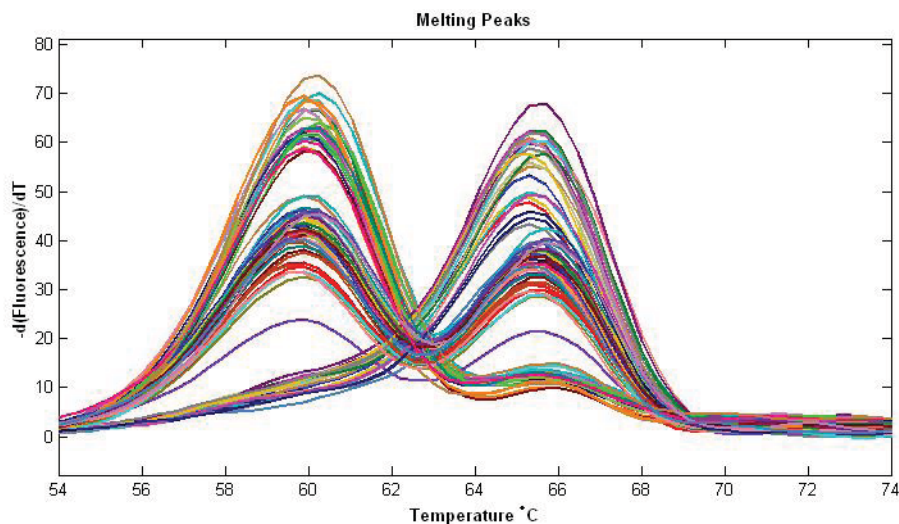
4. In the melting peaks graph, you should observe a completely flat region before and after the melting transition to get the best results.

If the cursors are located at the outer edges of the graph, pull them in towards the melting transition by dragging the cursors with your mouse or entering numerical values in the temperature boxes above the sliders.

Step 3. Grouping

The next step of the analysis is to compute the samples into groups based on the melting profile of the probe. In this tab, the data is presented in two graphs, the upper graph is directly carried over from the previous screen and can be toggled between melting curves and melting peaks. The lower graph displays melting peaks only. Also, the upper graph always displays all the samples in the analysis. The lower graph only displays user selected samples.
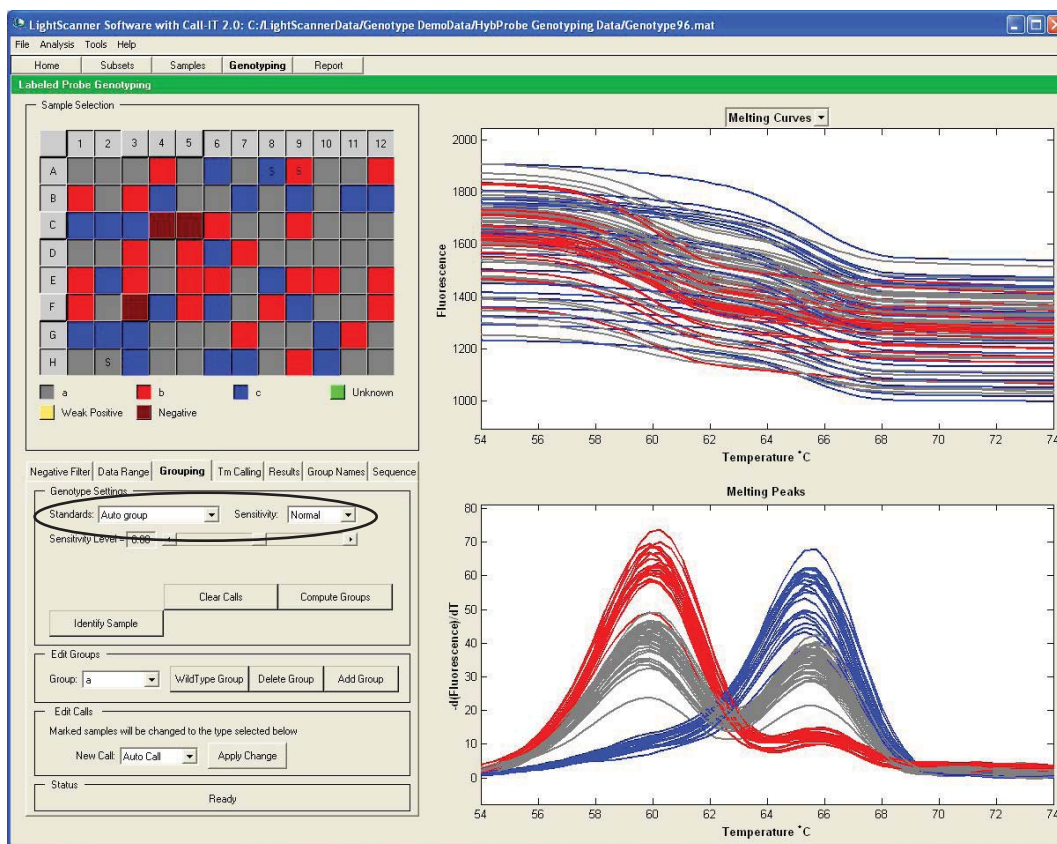
1. To compute the melting data into genotypes, first choose the grouping method from the **Standards** pull-down menu. The software offers three grouping options:

   - Auto Group—In this setting, samples are grouped by melting temperature with no bias towards any one group. Up to 10 unique groups can be assigned to a data set.
   - Use Internal Standards—in this setting samples are grouped around known standards defined by the user in the sample editor.
   - Use External Standards—in this setting the user has the option of importing external standards from a previous analysis for use with the current data set. Selecting the exter-

nal standards option will bring up a file selection dialog from which the user chooses the file containing the standards that will be brought into the analysis.
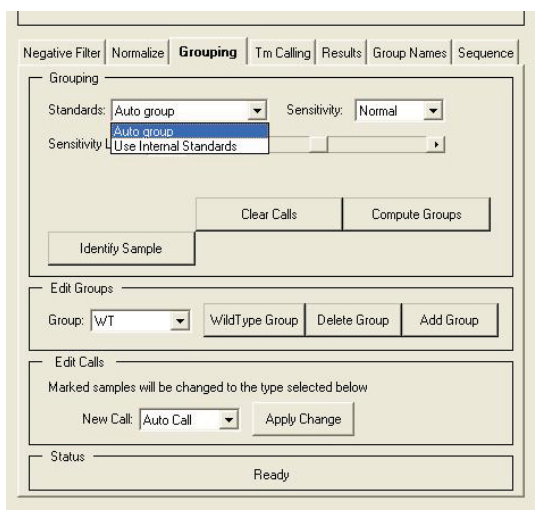
2. Choose the sensitivity. The software offers two sensitivity settings

   • Normal—Select Normal sensitivity for peaks that are separated by 3–5 °C.

   • High—Select High sensitivity to distinguish between groups that are separated by less than 3 °C.
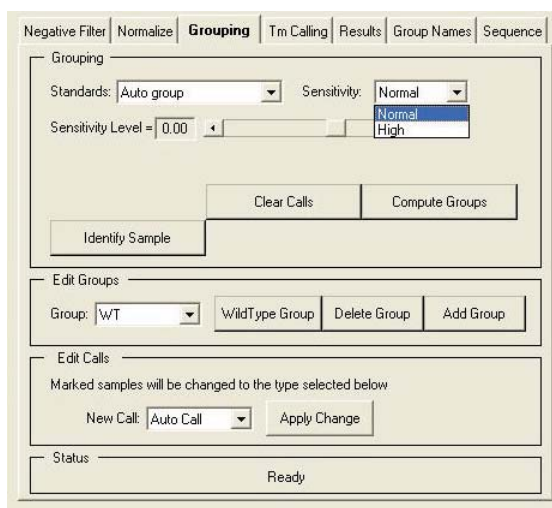
In general, normal sensitivity should be used for peaks that are separated by 3–5 °C. The High sensitivity setting may be needed for peaks that are less well separated.

3. Adjust the Sensitivity Level.

   Depending on the quality of the data you may need to adjust the grouping calls. The sensitivity slider bar allows you to adjust the grouping sensitivity to a value that best fits your data. The grouping call will change in real time as you adjust the slider, but you must select the Compute Groups button when you have found the desired setting to preserve that analysis. Finding the correct sensitivity is especially important when optimizing a new genotyping

experiment. Once the sensitivity is established for an assay, you should be able to use the same settings to analyze subsequent experiments.

- • The default (normal) sensitivity setting is zero.
- • To increase the number of groups, move the slider bar to the right or type in the desired value.
- • To reduce the number of groups, decrease the value in the slider bar.
- • For the most consistent results between experiments, set the sensitivity level such that an increase or decrease of 0.3 does not change the results. This will ensure that the calculations are performed in a robust sensitivity range and can be used in subsequent experiments.

4. Select the **Compute Groups** button to complete the analysis.

5. Samples will be color coded by group and displayed graphically as both Normalized Melting Peaks and Normalized Difference Curves. The groupings are displayed by color both on the graphs as well as the plate map for easy cross-reference.

6. If the Internal Standards grouping option was selected but only a single reference sample is used, all the samples that belong to the group containing the reference sample are grouped together. All the remaining samples are assigned as Unknown (green). To further cluster the unknown samples into individual groups, select the Group Unknowns button in the Settings box.

7. Save the results by going to the **File** menu and choosing **Save**.

Step 4. Tm Calling

The melting temperature (Tm) of a sample is defined as the point in a melting curve where 50% of the probe-target hybrid is dissociated. The LightScanner software is designed to automatically calculate the Tm for every sample.

To calculate the Tm automatically, select the **Automatic Mode** method, then do the following:

1. In the **Selection** box pull-down menu, select **Genotype**. This option groups samples by the genotyping assignments made in the previous screen.

2. Select **Normal** in the **Sensitivity** box if you only want to analyze the major peaks. The Tm may appear to be skewed due to contributions from minor peaks. Select **High** in the **Sensitivity** box if you want to analyze major and minor peaks independently.

3. Finally, select **Compute Tm Values**.

4. The results will be displayed in tabulated form in the Tm Calling Data display below the graph. For heterozygous samples, an additional calculation of ΔTm is also displayed.

5. The data table will display the following data:

   Tm1   The melting temperature of the first peak in the sample
   Tm2   The melting temperature of the second peak in the sample
   ΔTm   The difference in the melting temperatures between the 2 peaks

6. Data that the software automatically calculates but does not display includes peak area, height, and width and shoulder regions. This information can be displayed by selecting the check boxes in the Data Display frame.

Optional data	
Area 1	The area of the first peak in the sample
Area2	The width of the first peak in the sample
Width1	The area of the second peak in the sample
Width2	The width of the second peak in the sample
Height1	The height of the first peak in the sample
Height2	The height of the second peak in the sample

7. Data can be sorted by Group, Row, Column, Name, or Tm by clicking on the column headers.

8. Genotyping calls can be edited based on Tm assessment by using the Edit Calls menu. Right click + box the samples you wish to edit. Use the options in the pull down menu to select the new call, and select **Apply Change**.

9. A summary of all the results is compiled in the Results tab window. These results can be exported as a tab delimited file for data management purposes. To export the data, go to **File>Export>Genotyping Results** under the Menu Bar.

Manual Tm Calling

You can also determine peak area and Tm manually. Manual Tm should be calculated ***one sample*** at a time.

1. Select a single sample from the sample grid.

2. Under **Method** choose **Manual** mode. For Labeled Probe Analysis, the Tm Calling Editor below the graph now displays two check boxes if the peaks option is set at two peaks or less, and six check boxes if six peaks or less is selected. For Unlabeled Probe Analysis, two check boxes are displayed.

3. Checking the boxes immediately brings up a cursor on the screen that can be dragged to any position on the graph. The position of the cursor is indicated numerically next to the check box. This is the manually calculated Tm.

4. Tm values are computed in real-time, and the compute Tm values button is inactive.

View Tabulated Results

Move to the **Results** tab to view the analysis in tabulated format. Data is displayed by Row, Col, Name, and Grouping Call. If a sample has been manually reassigned to a group, it will be marked by an asterisk to flag it for future reference. These results can be exported in tab-delimited format (Excel compatible) by going to the **File** menu and choosing **Export Scanning Results**. A *.rst file will then be generated that can be opened with Excel or another spreadsheet program.

CHAPTER 10

LIGHTSCANNER TROUBLESHOOTING GUIDE

Troubleshooting Guide for Chemistry

Symptom	Probable Causes	Solutions
Low overall fluorescence (the sample is weakly fluorescent)	Plate map not aligned to wells of plate.	Use software alignment tool to align the map to the plate.
	Acquisition of data was started after melting transition of fragment had already begun.	Adjust melting window to accommodate the melting characteristics of fragment.
	PCR is not robust.	Assess robustness of PCR by gel electrophoresis. Adjust reaction chemistry ($MgCl_2$, primer concentration, etc.) or cycling parameters (annealing temperature, cycle number, etc.) to increase robustness of reaction.
	No oil overlay of single or scattered sample(s).	Set up PCR for those particular sample (along with needed controls) again.
	No oil overlay for entire plate.	Make sure the entire plate is equally dehydrated (e.g., 90 ºC for 10 min. on an uncovered thermocycler). Rehydrate samples by adding water and oil overlay. Remelt the samples.
	Nonoptically clear sealing film on the plate.	Replace with an optically clear film or remove altogether. See Appendix B for a list of optically clear plate films.

Symptom	Probable Causes	Solutions
Inconsistency of profiles between replicate samples or samples of common genotypes	Different DNA isolation chemistries (final elution buffer, storage buffer) used to prepare individual specimens.	Isolate DNA samples using a common protocol or bind existing DNA to silica and elute specimens into a common buffer.
	Components of reaction mixture not consistent between samples (e.g., $MgCl_2$ concentration, primer concentration, adjuvant, etc.).	Re-run with a consistent reaction composition among all of the samples.
	Presence of nonspecific amplification products in the reaction.	Assess reaction by gel electrophoresis. Optimize the PCR chemistry and cycling conditions as described in Chapter 3.
	Inconsistency of reaction volume between samples.	Re-run with a consistent reaction volume.
	Inconsistent template DNA concentration between reactions.	Adjust template DNA specimens to be at a common concentration and add equal amount to each reaction.
	Bubble present in a reaction well.	Centrifuge plate to remove bubble.
Unexpected/ Inconsistent low temperature melting inflections	Presence of nonspecific amplification product.	Assess reaction products by gel electrophoresis. Optimize the PCR chemistry and cycling conditions as described in Chapter 3. Use a Hot-Start method.
	Primer dimer product.	Evaluate primer design and re-design to eliminate dimer formation. Increase annealing temperature to make dimer formation less favorable. Reduce duration of annealing step to make dimer formation less favorable. Use a Hot-Start method.

Symptom	Probable Causes	Solutions
Unexpected/ Inconsistent high temperature melting inflection (toeing)	Presence of non-specific product having a higher melting temperature than the desired product.	Assess reaction by gel electrophoresis. If using a previously optimized PCR reaction, check for degradation of primer(s) by rediluting from a stock and reassess the reaction. Synthesize new primers if necessary. If using a nonoptimized PCR reaction, optimize the PCR chemistry and cycling conditions as described in Chapter 3.
PCR reaction fails when LCGreen® dye is included	PCR inhibition when using AmpliTaq® Gold or a home-brew Taq polymerase enzyme. Occasionally poor performance has been observed when using these enzymes with LCGreen Plus.	Try a different thermostable polymerase enzyme. The mechanism for the inhibition remains unclear. Recommended enzymes include: Klentaq™ (AB Peptides), Fast Taq (Roche), Taq DNA polymerase (AB Gene, GibcoBRL). Hot-start procedures, although not necessary for well-optimized reactions, are widely recommended because it is easier to obtain clean PCR products.
	Dithiothreatol (DTT) is present in reaction mixture. LCGreen when in the presence of DTT can cause the precipitation of bovine serum albumin (BSA). The precipitate is intensely stained by the dye.	Use a buffer that is missing either DTT or BSA.
	LCGreen dye raises the annealing temperature of primers and the denaturation temperature of PCR products.	Adjust annealing and denaturation temperature upward to reestablish optimal PCR.

Symptom	Probable Causes	Solutions
Unusual melting profile not observed with sample known to contain heterozygous sequence variation	A polymorphism resides under an amplification primer. A polymorphism under a primer may cause allele specific amplification leading to the variant of interest being either poorly represented or not represented.	Re-design the primer to avoid polymorphic sites.
	Differences in the melting profiles may be subtle.	Use Difference Plots to compare curves and make sure samples of the same genotype cluster well.
	Certain SNPs and single nucleotide insertions/deletions are more difficult to detect than others. Longer amplicons show less deviation from wild type than shorter amplicons.	Ideal PCR product lengths are 100–250 bp, although SNPs in over 1,000 bp products can be detected.
	Melting window is excessively wide.	Begin data acquisition 2–5 °C below the start of the melting transition so that differences are more readily observed.
Difficulty analyzing high Tm PCR products	For PCR products that melt at high temperatures, it may be difficult to completely melt the products and get adequate high-temperature background for analysis.	In this case, consider adding dimethyl sulfoxide ([DMSO] 5–15%), either before (preferred) or after the PCR.

Troubleshooting Guide for Hardware

Symptom	Possible Causes	Test	Solutions
Front panel Power LED does not turn on	LightScanner is turned off	Make sure power switch is in the on position.	
	LightScanner has no power	Cycle power to the instrument. Front door will open, and beep can be heard. LED fans can be heard humming for the first 5 sec.	If both occur the LightScanner has power, and the problem may only be with the LED.

The LightScanner will run without the LED. To repair the LightScanner must be returned. |
| | LightScanner is unplugged | Cycle power to the instrument. Front door will open, and beep can be heard. LED fans can be heard humming for the first 5 sec. | If neither occurs it is possible the LightScanner has no power.

Check that both ends of the power cord, and ensure both are firmly seated in their respective sockets. |
| | LightScanner fuses are blown | Cycle power to the instrument. Front door will open, and beep can be heard. LED fans can be heard humming for the first 5 sec. | If neither occurs it is possible the LightScanner has no power.

Check fuses; With in power switch in an off position and the instrument unplugged, remove fuse cartridge (light tan square, directly above the power switch). Wire in center of both fuses should be intact, and no burned or dark areas. Replace if necessary. |

Note: If you are still unable to operate the LightScanner instrument, please contact Idaho Technology's customer service department.

Symptom	Possible Causes	Test	Solutions
Run Software returns communications error	Serial cable is loose or unplugged.	Verify that the serial cable (9-pin) is firmly seated in its respective connector on the back of the LightScanner , and the serial port (Com Port 1) of the PC.	Re-seat cable.
	Software Conflict.	Open PC task manager and make sure no other programs are running that might be trying to access Com Port 1. Make sure multiple instances of the LightScanner Run Software are not running	Close extra application, and/or reboot.
	Serial cable damaged or broken Note: this is unlikely to occur	If a spare serial cable is available try using it.	Order a replacement cable from Idaho Technology.

Note: If the serial cable is properly seated, and you are reasonably sure there are no software conflicts, consult any possible software fixes/troubleshooting tips that may be available. If you are still unable to operate the LightScanner instrument, please contact Idaho Technology's customer service department.

Symptom	Possible Causes	Test	Solutions
Internal Blue Excitation LEDs do not turn on	Camera Cable loose or disconnected.	Verify that the camera cable is firmly seated in its respective connector on the back of the LightScanner and the PC. There should only be one connection on the back of the LightScanner and PC where this cable will fit.	Re-seat cable.

Note: If the camera cable is firmly seated in both the LightScanner and PC, follow any camera troubleshooting instructions you might have.

Symptom	Possible Causes	Test	Solutions
Melting Data has large spikes or fluctuations	Poor chemistry	Run test plate from ITI.	If ITI test plate looks good, the problem is likely to be from the end user's chemistry.
	Defective LightScanner	Run test plate from ITI.	If ITI test plate looks bad, the problem is likely to be from the LightScanner, and the instrument will need to be returned for repair.
	Bubble present in a reaction well.	Run test plate from ITI.	Centrifuge plate to remove bubble.

Returnable Problems

The problems listed below are indicative of a LightScanner hardware failure and are not diagnosed or repaired without the use of LightScanner Service Software and a trained technician.

If you encounter any of these issues or any undescribed failures, please contact ITI Technical Support for assistance.

- Door will not open/close.
- Temperature does not change.
- Lifter and ejector motors make abnormal noises or movements.
- Fans not turning on.
- Half of the plate image is very dim.
- Bar code reader not functioning.
- Blue excitation LEDs remain on after run is complete.
- Ejector will not return plate.
- Plate will not auto load.

APPENDIX A

FILE FORMATS

The LightScanner software creates various types of files as part of a LightScanner experiment. Each file is named using the experiment name, plus an extension that identifies the file type. Extensions include the following:

.mlt	A file containing the raw melting data from an experiment. The *.mlt file is created automatically when you run an experiment, as soon as the experiment is completed the software will perform a series of data pre-calculations that result in a *.mat file which should be opened to complete the analysis. A *.mlt file can also be opened in the analysis software however; the software will need to re-compute some of the calculations before the data file can be opened. Most users will prefer to save time by opening the *.mat file directly.
.mat	An analysis file generated after completion of an experiment. The *.mat file should be opened in order to complete the data analysis. Once the data analysis is complete you can save the file and retain all the analysis settings. You can also use the settings associated with a particular mat file to provide the settings for another experiment by importing analysis settings through the file menu. This allows you to apply a standard set of analysis parameters to all your experiments if required. (Note, negatives must be re-defined in every analysis, they are not imported with the settings.) ⚠ **Important: Do not open the file or adjust it in any way other than through the LightScanner software. Doing so can cause loss of data and other problems. You can rename the .mat file, if desired.**
.flo	A fluorescence file containing fluorescence data from a LightCycler® experiment. You can import a .flo file and perform an analysis of the data.
.rst	A result file containing the results of an analysis. The file is generated when you select **Save Results** from the LightScanner file menu after you compute genotypes.
.smp	A sample name file containing the names and locations of samples in your experiment. You can create the file, then import it to provide sample name information for the current experiment.
.tem	A temperature file containing the raw temperature data.
.cur	A tab-delimited file containing data for temperature and melting curve values. The first column in the file contains the temperature readings. The remaining columns contain the melting curve data in a column-oriented format: The second column contains fluorescence values for well position A1, the third column contains fluorescence values for well position B1, and so on.
.vs2	A fluorescence file containing fluorescence data from a HR-1™ experiment. You can import a .flo file and perform an analysis of the data.

APPENDIX B

LIGHTSCANNER PLATE AND SEALING FILM COMPATIBILITY

⚠️**CAUTION:** Use only white-well plates with the LightScanner. Clear plates allow fluorescence bleed over from well to well, and black plates quench fluorescence and increase noise.

💡 Note: Compatibility list is subject to change. Check ITI Web site for updates and additional information.

Manufacture	96-Well Plate	384-Well Plate	Sealing Film
Axygen www.axygen.com	PCR-96M2-HS-W[a]		UltraClear™ UC-500[b,c]
Bio-Rad www.bio-rad.com	HSP-9665[b] MSP-9651	HSP-3865[†] MSP-3852	Microseal® 'A' Film – MSA-5001 223-9444[b,c,d]
GeneMate by ISC BioExpress www.bioexpress.com	T-3078-W[d]	T-3157-W T-3109-W	
Applied Biosystems www.appliedbiosystems.com			ABI PRISM™ Optical Adhesive Covers – 4311971[b,c]
4titude® www.4ti.com.uk www.bioke.com	FrameStar® 964ti-0961 (Black frame white well)[b]	Framestar 3844ti-0385 (Black frame white well)[b]	
Phenix www.phenixresearch.com	MPX-96M2-W[a]		
G. Kisker GbR www.kisker-biotech.com	G060/H/IE-W[a]		
MBP www.mbpinc.com	3482W		
ABGene www.abgene.com	AB-0800/W		AB-1170[b,c]

[a]Compatible with the Applied Biosystems GeneAmp® 9700 Thermal Cycler (Please note that all plates compatible with the 9700 give a significantly reduced fluorescent signal)

[b]Preferred plate or sealing film

[c]Film may remain intact during melting analysis. (Please note that use of this film does not eliminate the need for an oil overlay.)

[d]Available by phone order only.

Compatible Mineral Oils

Manufacture	Catalog No.	Mineral Oil
Sigma www.sigmaaldrich.com	M5904	Mineral Oil Light for Molecular Biology
Fisher www.fishersci.com	0121-1	Mineral Oil Light
Alfa Aesar www.alfa.com	31911	Mineral Oil
USB www.usbweb.com	71600	Ultra Pure Mineral Oil - Molecular Biological Grade

⚠**IMPORTANT:** The use of mineral oil is required to prevent sample evaporation in the LightScanner.

APPENDIX C

PREVENTING CARRY-OVER (AMPLICON) CONTAMINATION

To achieve the best PCR results, it is essential to prevent amplicon contamination. There are both physical and chemical methods used to prevent contamination.

Physical Methods

Many thermal blocks are equipped with heated lids or covers which theoretically prevent sample condensation, evaporation, and loss during PCR cycling. To ensure sample volume retention and prevent contamination, all samples should be covered with a layer of mineral oil, and the plate should be sealed with a clear film or aluminum foil tape.

These measures are necessary for genotyping on the LightScanner for the following reasons:

1. The plate of reactions must be removed from the thermal block and spun down at 2000 rpm for 1–2 min. following PCR. This is a critical step, as air bubbles are often present following PCR and must be spun out of the reaction before analysis on the LightScanner. Some type of physical barrier is necessary when transporting the plate of amplified reactions from the thermal block and during the centrifuge spin.

2. The oil barrier helps reduce the risk of amplicon contamination and prevents the amplicon sample from evaporating. There are several brands of film that are optically clear, which may be left on the plate during melting. Use of these optically clear films is recommended, because they do not have to be removed during melting, thereby reducing amplicon contamination risk.

Chemical Methods

PCR is capable of amplifying a single molecule over a million fold. Even the most meticulous laboratory can experience some degree of amplified product carry-over into the "clean" PCR setup area. Amplicon contamination risk can be minimized by adding uracil-DNA glycosylase (UNG) to the reaction mix if dUTP has been substituted for dTTP. UNG catalyzes the removal of uracil from single- and double-stranded DNA that has been synthesized in the presence of dUTP (but does not degrade native DNA or PCR primers). Therefore, UNG is only effective in contamination prevention if dUTP was substituted for dTTP at the inception of the assay.

APPENDIX D

RETURN PROCEDURES

An instrument problem may be reported by calling, faxing, e-mailing, or mailing Idaho Technology. The contact numbers are located below.

If you are returning an instrument for repair, follow the basic guidelines prescribed in this appendix. You will also need the following documents:

Declaration of Decontamination
Decontamination Labels

These forms and instructions can also be downloaded from **http://www.idahotech.com/product_sup/index.html.**

Phone

(800) 735-6544 - United States and Canada
(801) 736-6354 - Utah
+1-801-736-6354 - International

Fax

(801) 588-0507 - United States and Canada
+1-801-588-0507 - International

E-mail

support@idahotech.com - Technical Support

Web Site

http://www.idahotech.com/product_sup/index.html

Address

Idaho Technology, Inc.
390 Wakara Way
Salt Lake City, UT 84108
USA

Step 1: Obtain a Return Materials Authorization Number

The first step in returning an instrument to ITI is to obtain a Return Materials Authorization (RMA) number from our Service Department. You can either call Technical Support at 1-801-736-6354 or 1-800-735-6544 or complete the supplied fax forms (two pages) and fax them to ITI. A Technical Service specialist will contact you with an RMA number and further instructions. If your instrument is still under warranty, please supply the purchase date and serial number, if your instrument is out of warranty, please supply a blank PO# for the repair charges.

Step 2: Decontaminate All Returned Equipment

You must decontaminate all equipment being returned to ITI to remove amplicon contamination and to ensure that personnel handling the equipment are not harmed by pathogenic organisms.

The person responsible for the return must thoroughly decontaminate the unit by following the instructions below and in "Cleaning and Decontamination," of Chapter 1, Installing the LightScanner Instrument.

Chemical Lab Decontamination

If a chemical lab makes the return, the customer must thoroughly clean chemical contamination from the instrument.

Biological Lab Decontamination

If the instrument was used in a clinical lab, the person responsible for the return must thoroughly decontaminate the unit by wiping it down with a 10% bleach solution (chlorine bleach diluted 1 part bleach to 9 parts water), followed by wiping it down with distilled water to remove any contaminants. Be sure not to mix the bleach solution with other chemicals (mixture with acid will liberate chlorine gas). Since the solution is caustic, gloves and a face shield are recommended when preparing and using the solution. When corrosion by the 10% bleach solution may be a problem, flush with water is permissible after the 10% bleach solution has remained in contact for a minimum of 10 min.

Step 3: Decontamination Labels

After the above steps have been completed, the person responsible for the return must complete and sign two decontamination labels and attach one to the instrument and the other to the exterior of the shipping container. Complete and sign the Declaration of Decontamination form; a photocopy should be made for your records and the original must be returned with the instrument.

Note: An RMA number must be obtained from ITI before shipping. The RMA number and decontamination labels must be visible on the exterior of the shipping container.

ITI reserves the right to return or refuse receipt of any materials at the customer's expense that do not meet these requirements.

Step 4: Packaging and Shipping

1. Package the instrument inside a large heavy weight box.
2. Put all extra components in a separate box and place the instrument into the box.

3. Put the accompanying computer and monitor in a heavy weight box, seal it with packing tape, and place it in a shipping box.

4. Fill all sides and top of the shipping box around the instrument with at least two inches of instant packing foam, or packaging material for protection against shipping damage.

5. Seal the box with packaging tape.

International Shipping

If an instrument is shipping internationally, the instrument MUST have an export license.

RETURN MATERIALS AUTHORIZATION FAX   PG. **1** OF **2**

This form may be faxed to our return center at 1-801-588-0507 (U.S. and Canada) or +1-801-588-0507 (International). It must also be included with the returned instrument.

If your equipment is still under warranty, please supply the purchase date and instrument serial number. If your instrument is out of warranty, please supply a blank PO# for the repair and shipping charges. Please note that your item must be returned with a ITI assigned RMA# and decontamination documentation.

From: _____   Pages: _____

Attn.:_____

Ship to: Idaho Technology Inc.   Warranty:_____
                390 Wakara Way
                Salt Lake City, UT 84108   Nonwarranty:_____
                USA

Date: _____

RMA#: _____   (required when returning an instrument)

Company: _____

Department: _____   PO#: _____

Phone: _____   Fax: _____

Model: _____   Serial No. _____

Laptop Login and Password (needed to QC test the LightScanner system): _____

_____

Notes:_____

_____

_____

The decontamination form and labels must be completed before the instrument is returned to Idaho Technology. If the unit is returned without decontamination documentation, it will be shipped back to you at your expense. Nonwarranty charges are standard labor charges with a 2-hour minimum charge plus parts and shipping charges. If the unit cannot be repaired, it will either be returned to you or disposed of upon your request. Thank you for your business and for supporting the health and well being of our employees.

Signature:_____   Date: _____

RETURN MATERIALS AUTHORIZATION FAX

Declaration of Decontamination

This instrument has been decontaminated according to established Idaho Technology Inc. biological decontamination procedures.

Which method was used? _____

_____

_____

What chemical, infectious, toxic or radioactive substances have been in contact with this product? (Also indicate if flammable or corrosive.)_____

_____

_____

Authorization Notice

By accepting this authorization to return this product, the user assumes all responsibility for decontamination and cleaning. Idaho Technology Inc. reserves the right to refuse the delivery of products, which do not appear to have been properly decontaminated. If the equipment was used with or around radioactive material, the signature of the safety officer is also required.

Signature:_____ Date: _____

Decontamination Labels

Please complete these Decontamination Labels and affix one to the instrument case and the other to the exterior of the shipping carton. Failure to decontaminate before shipping to ITI will result in the immediate return of the unit at your expense.

Copy and cut out the label and attach it to the case of the instrument being returned.

DECONTAMINATION NOTICE:

This Product has been decontaminated per Return Equipment policy: _____

Idaho Technology Return Authorization (RMA#):_____

Customer Name: _____

Customer Address:_____

_____

Signature: _____ Date: ____/___/____

Copy and cut out the label and attach it to shipping container of the instrument being returned.

DECONTAMINATION NOTICE:

This Product has been decontaminated per Return Equipment policy: _____

Idaho Technology Return Authorization (RMA#):_____

Customer Name: _____

Customer Address:_____

_____

Signature: _____ Date: ____/___/____

APPENDIX E

ADDITIONAL OPTIMIZATION CONSIDERATIONS

Reaction Buffers

Hi-Res Melting may be performed in any standard PCR buffer. The PCR buffers offered by ITI provide some advantages. These buffers do not contain KCl, which may be beneficial when amplifying fragments (usually below 400 base pairs) frequently assayed by Hi-Res Melting. The second advantage ITI buffers offer is that they are provided in 10X $MgCl_2$ concentrations of 10 mM, 20 mM, 30 mM, 40 mM, and 50 mM. Buffers having different concentrations of $MgCl_2$ are useful for developing and optimizing new or existing PCR reactions. Changing the magnesium concentration is achieved with alternative buffers. Intermediate concentrations may be obtained by mixing buffers. For example, if a final concentration of 1.5 mM $MgCl_2$ is required, equal volumes of the 10X buffers containing 10 mM $MgCl_2$ and the 20 mM $MgCl_2$ may be combined to generate a 10X buffer with 15 mM $MgCl_2$ that provides a working concentration of 1.5 mM $MgCl_2$. Without experimental data, it is impossible to determine what $MgCl_2$ concentration will work best with a new primer set. Having pre-made buffers with a range of $MgCl_2$ concentrations is convenient.

Deoxyribonucleotides (dNTPs)

It is recommended that dNTP's be used at 200 µM each. An acceptable substitute for dTTP is dUTP. When dUTP is used, the concentration is typically 600 µM (opposed to 200 µM dTTP). Fragments containing deoxyuracyl melt at a lower temperature compared to the same fragment containing deoxythymidine.

Taq Polymerase

For Hi-Res Melting applications, hot-start procedures are recommended. Options for hot-start include antibody-mediated hot-start and thermal activated enzymes.

Oligonucleotide Primers

High performance liquid chromatography (HPLC) purified primers are recommended. Where to obtain oligonucleotides is based upon personal preference and the history of individual laboratories. IT BioChem, a division of Idaho Technology, provides current good manufacturing practice (cGMP) HPLC purified primers that perform well in Hi-Res Melting. Properly quantifying primers is critical to effective amplification. Beginning with the guidelines provided by the manufacturer, resuspend primers at 1.5- to 2-fold higher than your desired concentration. Measure the concentration by spectrophotometry and adjust accordingly. Resuspend primers in buffer such as MOPS (Tris, 3-[N-morpholino] propanesulfonic acid), or HEPES (4-[2-hydroxyethyl]-1-piperazineethane-sulfonic acid) used at 2–10 mM. The pH should typically be 7.1–8.3. So-called TE buffer (10 mM Tris, 0.1 mM EDTA [ethylenediaminetetraacetic acid]) is acceptable.

Primers containing 5' GC clamps are frequently employed with presequencing gene analysis platforms such as single-strand conformational polymorphisms and dHPLC. The use of GC clamps has not been demonstrated to provide any benefit in Hi-Res Melting.

Genomic DNA

Hi-Res Melting compares profiles from independent PCR reactions; therefore, minimizing reaction-to-reaction variability is pivotal. To minimize variability, standardize the template DNA by

- Quantifying DNA samples using spectrophotometry
- Using a common buffer for all samples
- Adjusting samples to a uniform concentration
- Using the same amount of template in each reaction

For Hi-Res Melting, 10–30 ng of template DNA in a 10 μL reaction volume is recommended. Archival genomic DNA (material stored frozen for an extended period) presents unique challenges. Sublimation, the direct transition of frozen material to gas, frequently occurs in archived samples, and concentrates the DNA, salts, and other material that affect both amplification and Hi-Res Melting. Repurifying archived DNA by binding the DNA to silica and eluting it into a fresh buffer improves the quality and leads to better PCR and Hi-Res Melting. Numerous kits are available to purify DNA, and their performance is largely equivalent. Gel filtration chromatography is also acceptable for repurifying DNA.

Optimizing Thermal Cycling Conditions

Annealing Temperature

The use of gradient PCR is a useful tool for quickly optimizing PCR cycling conditions. Annealing temperature is the aspect of thermal cycling having the most influence upon specificity and robustness of amplification.

The figure below demonstrates the necessity of determining an appropriate annealing temperature for generating robust and specific PCR. Gradient PCR was used to amplify a 319 base pair fragment containing the 4th exon of the human *SCARB*1 gene. The software that designed the primers indicated the forward and reverse primers to have Tm values of 58.6 and 58.2 °C, respectively. Two primer concentrations were tested: either 0.2 μM of each primer or 0.3 μM of each primer. Common to all reactions was 1X buffer, 1X LCGreen Plus, 200 μM each dNTP, 2 mM $MgCl_2$, and 1 unit of Klentaq polymerase in a complex with TaqStart® antibody. Cycling conditions were as follows:

Cycle	Time	Temperature
Target temperature	1 min	94 °C
45 cycles	10 sec	94 °C
Annealing	30 sec	60–72 °C
	10 sec	72 °C
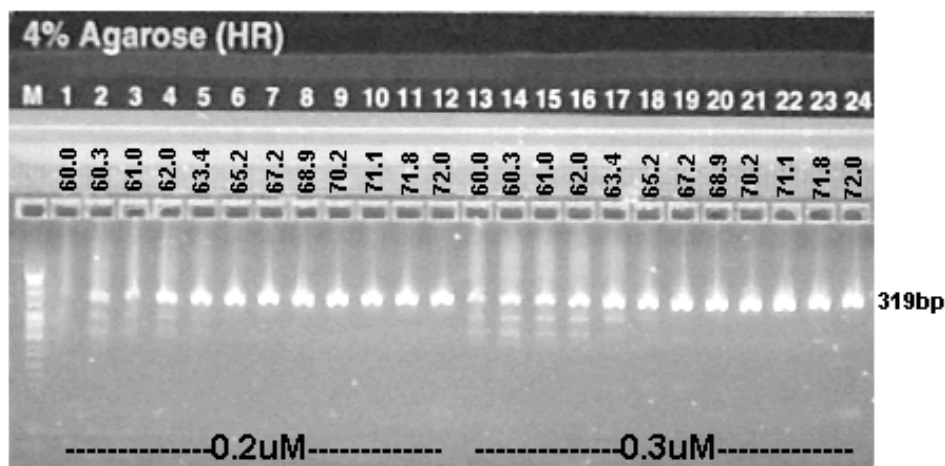
Reaction product was analyzed by gel electrophoresis using a 4% agarose gel. Lanes 1–12 analyzed reactions containing 0.2 µM of each primer, while lanes 13–24 analyzed reactions containing 0.3 µM of each primer. At low annealing temperatures (lanes 1–3 and 13), the specific product is poorly represented, and undesired amplification products are observed. As the annealing temperature increases the 319 base pair, specific product becomes more prominent until it is the only product observed (lanes 6-12 and lanes 20–24). Specific product is observed between 65.2–72 °C using 0.2 µM each primer and between 67.2–72 °C using 0.3 µM each primer. Note that robust product is observed at 72 °C, which is 14 °C beyond what the software predicted to be the Tm of the primers. Salts, LCGreen PLUS dye, and adjuvant (DMSO,` glycerol, etc.) also affect Tm.

If a thermal cycler with gradient capabilities is not available, a simple option is to begin by using conditions that will not overly tax the primer set.

1. Begin with a 10 µL reaction using the following:
   - 1X reaction buffer
   - 2 mM $MgCl_2$
   - A relatively high concentration of each primer (0.3–0.5 µM)
   - 1X LCGreen Plus
   - 10–30 ng of template DNA
   - Hot start enzyme (investigators' preference of enzyme and hot-start mechanism)

2. Choose cycling conditions where the annealing temperature is at or slightly above the average estimated Tm of the primers.

3. Place a 1–2 min hold at 94 °C at the beginning of the amplification program and use 35–45 cycles of PCR.

4. Recover samples from the plate and assess them by gel electrophoresis. Based upon the outcome of the initial experiment, it may be necessary to change the annealing temperature.

The amount of specific product, the presence or absence of undesired product, and the presence or absence of dimer product will dictate to the investigator how to best proceed in optimizing the reaction. If undesired product is observed, the annealing temperature may be increased. If amplification is not robust, the annealing temperature may be decreased and/or the duration of the annealing step may be increased. To complete the optimization process, use the following steps.

1. **Extension Phase:** The extension step in the PCR cycling protocol may be adjusted. In Hi-Res Melting, the fragments assayed are typically short (500 base pairs or less). Extending fragments of these lengths requires a hold at 72 °C for 5–20 seconds. If the annealing temperature in the cycling program is 65 °C or above, it is often possible to eliminate the extension step altogether. In the figure above, the range of temperatures, where only the specific product is observed (65–72 °C), would likely enable the extension step to be eliminated. Two-temperature cycling conditions (2-step PCR) promote specific amplification with the added benefit that programs run in less time.

2. **Cycle Number:** Increasing or decreasing the number of PCR cycles is useful to generate specificity and robustness in the amplification. If the PCR is robust with 45 cycles of PCR, lowering the number of cycles may be an option. At times increasing the number of cycles is useful to make amplification more robust. When PCR was in its infancy, it was believed that cycling more than 30–35 times would promote amplification of undesired products. There is some truth to this belief, but it is not entirely correct. In the past, it was not uncommon to use 100–500 ng of template DNA per reaction where today 5–20 ng is standard. Excessive template DNA will cause undesired product to be amplified the same way excessive primer concentration will lead to amplification of undesired product. Additionally, the quality of oligonucleotide primers has improved dramatically making amplification more specific and robust. Current Taq polymerases show superior and reliable processing compared to enzymes 10 years ago. All of these features mean more reliable PCR. Also, using more cycles (40–50) may be done without overt fear of creating undesired product. Reluctance to use more PCR cycles results from the time when the overall process of PCR was less understood and both reagents and instruments were less advanced than those available today.

Optimizing Internal Reaction Chemistry

Primer Titration

 Depending on the amount of specific product observed in the initial experiment, run a dilution series of your primers. If the specific product is robust, try titrating the primers downward. If the specific product is weak, try titrating the primers upward. Use 0.1 µM increments in the titration.

Determining Magnesium Chloride Concentration

Adjusting $MgCl_2$ concentration helps improve the specificity and robustness of PCR. If dimer product or undesired product is observed, decrease the $MgCl_2$ concentration. This makes conditions more stringent. If undesired product is not observed, increase the $MgCl_2$ concentration to increase robustness. When PCR is performed in capillaries, it is not uncommon to use up to 5 mM $MgCl_2$ but this concentration is rarely used when PCR is performed in block style thermal cyclers. While the use of $MgCl_2$ above 2.0 mM is infrequently seen when PCR is performed in thermal blocks, our observations indicate that higher $MgCl_2$ concentrations can be used to create more robust amplification. It is worth the effort to try higher $MgCl_2$ concentrations with some reactions.

The amount of $MgCl_2$ in a reaction will affect the shape and Tm of the melting profile. We have observed that some fragments that melt with a single inflection at low $MgCl_2$ concentration may

melt with multiple inflections at high MgCl$_2$. While this was observed with a small number of fragments, investigators should be aware of this possibility. As a general rule, in a higher MgCl$_2$ concentration, a fragment will melt with a slightly higher overall Tm.

Addition of Adjuvants: To obtain robust and specific PCR, the user may want to use an adjuvant in the PCR reaction. We have used LCGreen Plus with DMSO, TMSO (tetramethylsulfoxide, sulfolane), KCl, and glycerol. Currently, these reagents have not affected fluorescence intensity, altered dye binding characteristics, or made it difficult to detect sequence variation.

DMSO is commonly used to amplify regions of high G:C content by relaxing the stability of the hybrid. The adjuvant is typically used at 5–15% of reaction volume.

TMSO helps enhance specificity and robustness of PCR. The precise mechanism for the reaction is not well understood, but its efficacy can at times be quite remarkable. TMSO is solid at room temperature but liquefies when incubated at 40 °C. Liquefied TMSO is diluted in water (10–30% stock solution) and typically used at or below 3% in the PCR reaction cocktail.

The salt KCl enhances amplification and has been extensively used to optimize multiplex reactions. Some PCR buffers come with KCl, while others (such as Idaho Technology buffers) do not. The typical 1X concentration of KCl is 15 mM, but this can be significantly increased (up to 5 fold), decreased, or eliminated altogether. Increasing KCl may be used to enhance amplification of short products (<400 bp) while reducing KCl may enhance amplification of longer products (>600 bp).

Developing a Panel of PCR Assays to Function Together in Hi-Res Melting

Using Hi-Res Melting, the LightScanner instrument can also rapidly assess an entire gene for sequence variation. To assess multiple fragments, it is necessary to develop a common PCR condition and melting protocol that accommodates all of the individual fragments. The figure below shows part of the process to develop an assay for the human N-acetylglutamate synthase (NAGS) gene. The gene contains 7 exons, but exon 1 was assayed as 2 overlapping fragments, hence the 1a-1b nomenclature.

Panels (A) and (B) show gradient PCR for the exon 1a fragment and exon 5 fragment. Similar experiments were performed with the remaining 6 fragments of the panel. A compromise cycling condition of 1 min. at 94 °C, 45 cycles of 10 sec. at 94 °C, and 30 sec. at 70 °C was determined. Internal reaction chemistry for individual PCR reactions was optimized.
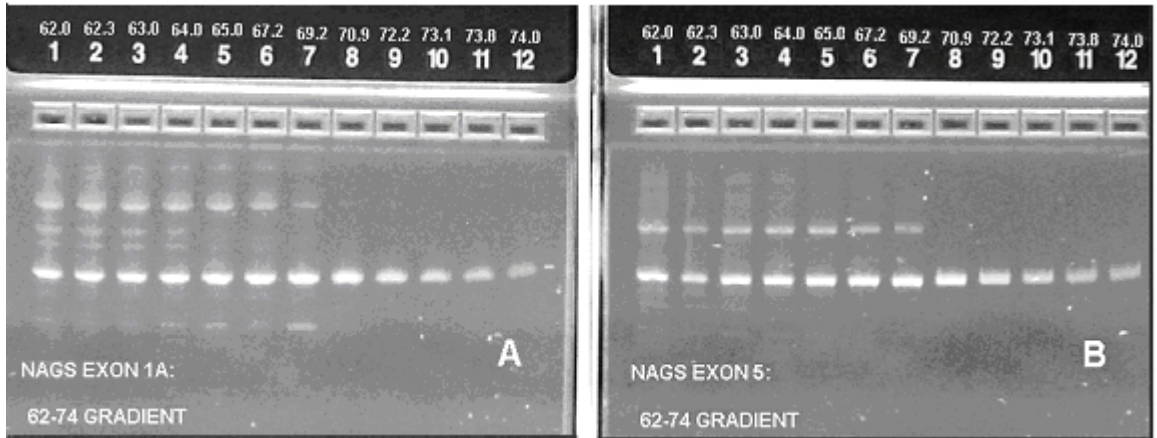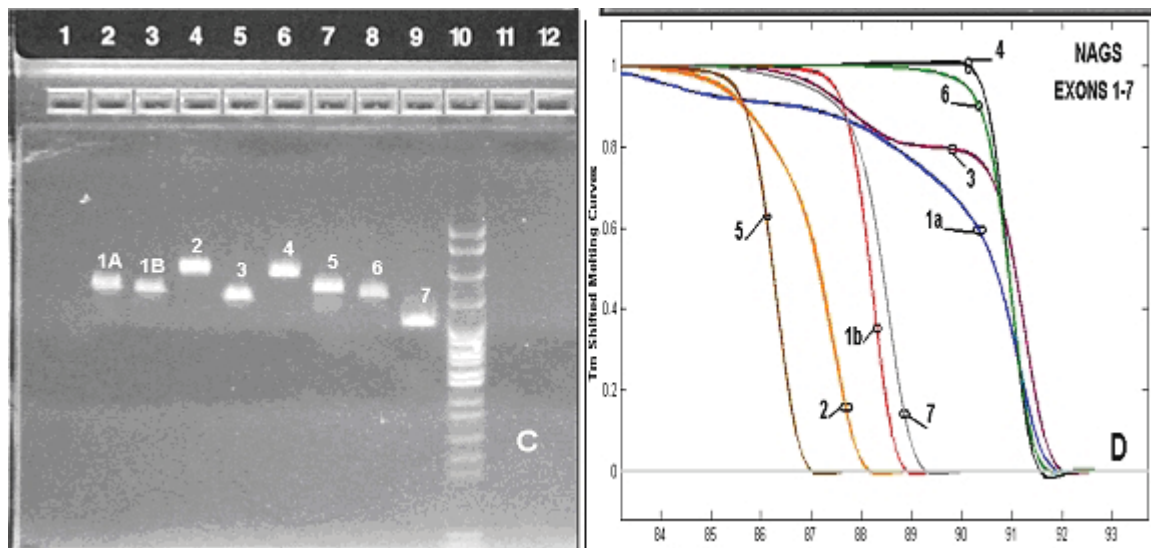
Panel A

Panel B

Panel (C) shows PCR products for all 8 fragments using the compromise cycling protocol and optimized internal chemistry for each reaction. All reactions are robust and contain only the specific product. After melting each fragment separately, a melting window was chosen that would accommodate all 8 fragments. In this case that melting window was 83–94 °C.

Panel (D) shows the melting profiles for the 8 fragments used in the NAGS assay panel. The 8 profiles in panel (D) are in fact profiles of 12 specimens.

Panel C

Panel D

Using LightCycler and HR-1™ (optional, Part No. HR01-ASY-0001): The LightCycler and the single sample HR-1 Hi-Res Melting instrument are useful tools for assay development. The real-time PCR curves generated by the LightCycler are useful for optimizing PCR reactions, but are not an acceptable replacement for gel electrophoresis. Differences in amplification efficiency that are not readily observed in an ethidium bromide stained gel are obvious in the real-time curves provided by the LightCycler. Amplification protocols in the LightCycler are fast compared to what is possible with a block-style cycler, which expedites assay development. PCR in a LightCycler and a block-style cycler are different, but despite this, conditions determined in a LightCycler may directly transfer to the block-style cycler.

The HR-1 is useful for determining the characteristics of a fragment so that the shape of the curve and melting points are known before large experiments in the LightScanner are carried out. The use of a LightCycler and HR-1 save time and labor but are not required.

Secondary Uses of PCR Product Previously Employed for Hi-Res Melting

Unlike other methods for pre-DNA sequence gene scanning, the PCR product used for Hi-Res Melting may be recovered for subsequent forms of analysis. The ability to recover the PCR product and use it in other types of analysis is unique to Hi-Res Melting. Following melting, the PCR product may be used for agarose gel electrophoresis or DNA sequence analysis. In both cases, the PCR product is handled the same as any other PCR product being used for these purposes. To use the PCR product as template for DNA sequencing, the product must be purified to remove primers and dNTPs. Essentially any standard procedure is applicable. The dye LCGreen Plus does not interfere with sequencing reactions or the subsequent sequencing read. For analysis by gel electrophoresis, tracking dye is added and the sample analyzed with an agarose or polyacrylamide gel. LCGreen Plus does not alter mobility or interfere with ethidium bromide staining. While no experimental evidence exists concerning subcloning of dye-stained PCR product, there is no reason to suspect these fragments could not be cloned into plasmids or other vectors.

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