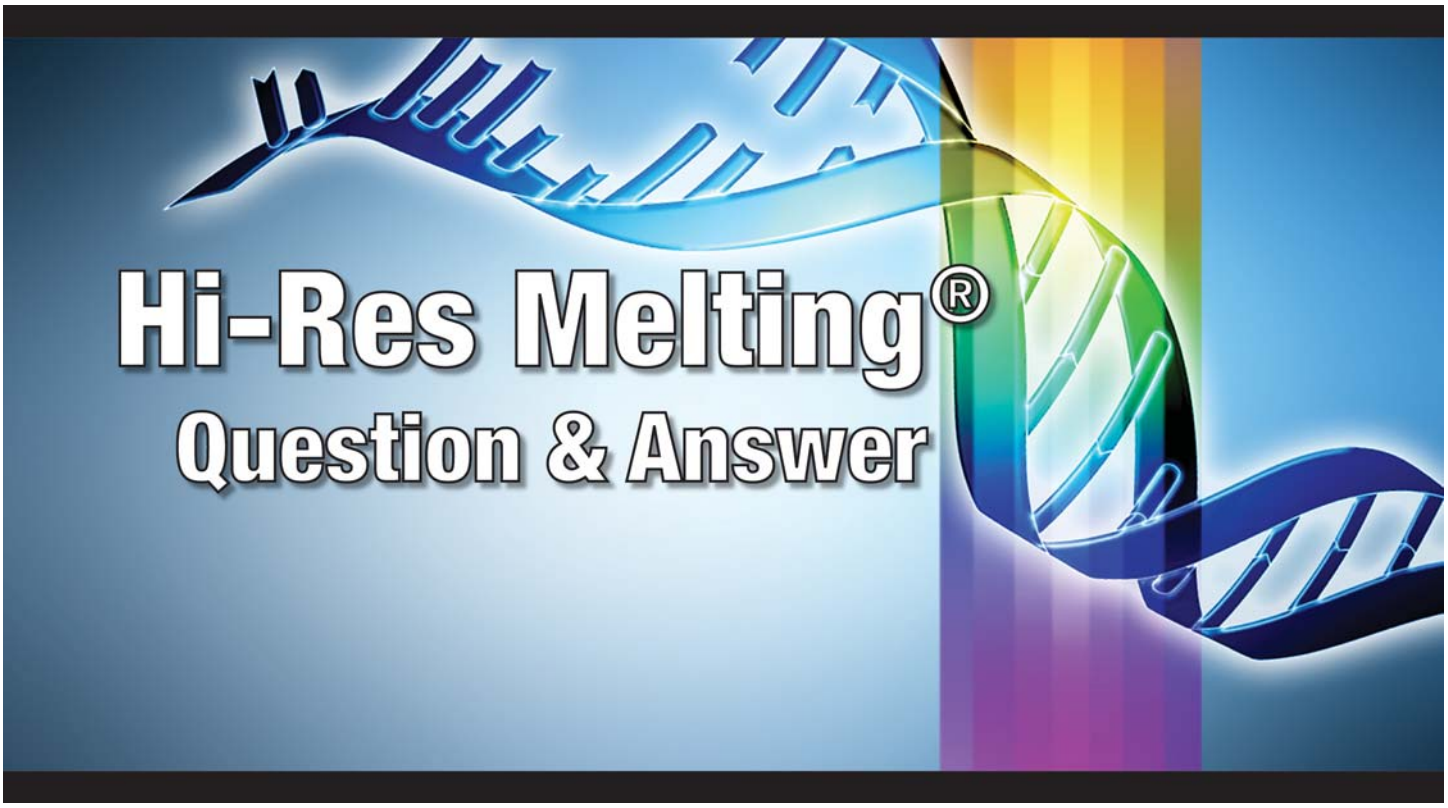

Idaho Technology Inc.

Designed for Hi-Res Melting® Idaho Technology Inc.

The information listed below represents the collective thoughts of Idaho Technology scientists, researchers, and support specialists, and is meant to only be used as a tool to assist current Idaho Technology customers. Should additional questions or concerns arise from the information listed below, please don't hesitate to contact Idaho Technology Technical Support at ITSupport@idahotech.com.

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Hi-Res Melting® Question and Answer

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CONTENTS

HI-RES MELTING QUESTION AND ANSWER

LightScanner and Other Technologies

Q: What are the differences between high resolution melting on the LightScanner and melt curve analysis on real-time PCR platforms?

A: Idaho Technology Inc, the inventor of high resolution melting (Hi-Res Melting®), enables DNA melting analysis with instruments and chemistries designed specifically for Hi-Res Melting, whereas most other technologies only offer an add-on melting module for specific PCR product confirmation. The LightScanner® instrument detects subtle differences in fluorescent signals over temperature change in 96- or 384-well plate format. The LightScanner was designed to be used in combination with LCGreen® dye, which is manufactured exclusively by Idaho Technology, Inc., and is unique in its ability to detect the presence of heteroduplexes formed during PCR. Other commercially available real-time PCR instruments lack the precise temperature control, the data density acquisition, and the analysis software required for successful implementation of the Hi-Res Melting method.

PCR Optimization

Q: Do I have to re-optimize my PCR reactions when adding LCGreen® Plus dye to my master mix or when I use any LightScanner Master Mix?

A: Yes. The addition of LCGreen dye to the PCR reaction and its presence in any LightScanner Master Mix stabilizes the double-stranded DNA molecules such that the Tm is elevated at least 1–3°C. We recommend performing a gradient PCR experiment to determine the optimal PCR conditions when using LCGreen Plus dye or any LightScanner Master Mix. From that point, it may be beneficial to experiment further with Mg titrations, primer concentrations titrations, and the number of PCR cycles.

Q: How can I reduce the Tm of my amplicon so that it can be analyzed on the LightScanner?

A: We recommend the addition of 5–15% DMSO to your PCR reactions to lower the Tm of all DNA in your PCR reaction. Please keep in mind that each PCR reaction must be re-optimized with the addition of DMSO.

Q: Can I analyze samples that were preserved and extracted by different methods?

A: Samples preserved and extracted by different methods can be melted on the same plate, but they should be analyzed in different subsets with their own respective controls. Characteristic differences have been noticed between different extraction methods such as formalin-fixed paraffin-

embedded (FFPE) extracted samples and cell-line extracted samples. Diluting by 2- to 10-fold, provided sufficient DNA quality and quantity, may be helpful in normalizing profiles between samples extracted via different methods.

Q: I am seeing a small peak that looks like a bump at the end of my melt profile. What can this bump be attributed to, and how can I eliminate it?

A: The bump is classified as a “toe” and is a classic sign of inefficient PCR. A toe most commonly corresponds to co-amplification of undesired PCR products that contain a higher melting domain than the amplicon. To increase the quality of your PCR reactions try one or a combination of the following:

- Double check sequence specificity of primers using some means such as an in-silico PCR tool.
- Re-evaluate primer concentrations to ensure accuracy.
- Decrease primer concentration by 20%.
- Increase annealing temperature by 1–2°C.
- Standardize DNA template concentration.
- Minimize input of DNA to no more than 20 ng total.

Variant Detection

Q: Our LightScanner assays are generating false positives. Am I doing something wrong?

A: No not necessarily. Hi-Res Melting has proven to be just as sensitive, if not more sensitive, than sequencing. It is not uncommon for Hi-Res Melting to detect a SNP that was missed during sequence analysis. Most mutations not initially identified by sequencing are those of somatic origin present at levels not practical for sequencing. We would recommend resequencing and manually sequence data analysis for confirmation of all false positives. Additional reasons for false positives are as follows:

- Insufficient PCR optimization—An aberrant peak at the end of the melt typically caused by co-amplification of undesired products is commonly dubbed a “toe.” It can result in an increased false positive rate due to its effect on the post-melt normalization region used and the level of curve shifting. Increasing the level of curve shifting >.05 may help when toes are present, but only if the toe region can be effectively dealt with during normalization. The best solution is to optimize the toe OUT of the PCR reaction by decreasing primer concentration and increasing the annealing temperature.
- Reduce potential pipetting errors—Normal pipetting variability may be more “visible” with Hi- Res Melting in the form of starting template concentration variability. Normally up to 3- to 4-fold concentration differences are invisible, but even 2-fold differences can be observed in Hi- Res Melting if there are other issues with the DNA storage buffer or if the amplicon is in a very difficult region, i.e., high GC content.
- Sensitivity settings during grouping require further adjustment—Reduce the sensitivity level so that all normal or wild type samples are consistently grouped and classified as nonvariants. It may be beneficial to experimentally optimize sensitivity settings by determining the typical melt profile variance of known wild-type samples that contain no mutations so that any variation from the typical wild-type melt profile can be further investigated.
- DNA extraction procedure standardization—Analyze samples prepared and extracted by similar techniques in the same subsets. For example, samples obtained from FFPE and cell lines should not be included in the same subset for comparison because each preparation technique produces characteristic differences in melt curve profiles.

Q: How does sensitivity and specificity affect variant detection during data analysis in LightScanner software?

A: It is important to note that sensitivity (false negatives) and specificity (false positives), as each relates to sensitivity settings in the software, are inversely related. As sensitivity is increased (as may be required to detect all known variants, particularly in larger fragments), the rate of false positives goes up, regardless of the technology or platform. The user must determine which is most important (sensitivity or specificity) and adjust data analysis sensitivity settings appropriately. If 100% sensitivity is desired, a higher rate of false positives is inevitable. If 100% specificity is desired, a higher rate of false negatives is inevitable.

Q: Replicate PCR reactions of the same sample produce different Hi-Res Melting results. Is there something wrong with my LightScanner?

A: Any variance in PCR product quality can yield different melting profiles, which may lead to differing results between PCR replicates. Variance in PCR quality is not uncommon and typically can be attributed to extraction method, integrity of reagents, pipetting errors, and the lack of reaching PCR plateau phase. Ensure that all replicates were extracted via a similar extraction technique, reagents are not expired, and all pipettes are calibrated. To assess plateau phase, either review real-time PCR data to ensure amplification has reached the plateau phase and has ceased before melting, or compare the intensity of PCR replicates on a gel while varying cycle number to ensure equal intensity across all replicates of a single cycle number.

Data Analysis

Q: When I try to open the LightScanner software nothing happens. How can I get the software to start up?

A: It may be possible that the display resolution for the monitor was altered. To set the resolution, do the following:

1. Right-click on the Desktop and select **Properties**.
2. In the **Display Properties** window select the **Settings** tab and set the screen resolution to 1280 x 1024.
3. Exit the Properties window and reboot the computer.
4. Restart the software.

If this does not correct the problem please contact Technical Support.

Q: Can the LightScanner software be operated on a Macintosh computer?

A: No. Currently Idaho Technology does not support Macintosh compatibility.

Q: What is the best way to set the normalization cursors during data analysis?

A: While performing linear or exponential background correction, the normalization cursors should be set such they are not within 0.5°C of any melting domain. When using linear correction, the area between each set of cursors must contain melt curves of the same slope (parallel lines), and the normalized curves in the lower window should be level just before and after each cursor set.

Q: What determines whether I should use linear or exponential background corrections?

A: Linear correction is suggested for use when a single melt domain is observed. Exponential correction is suggested when multiple melt domains are observed and when linear correction does not produce optimal results.

Q: When I open LightScanner software on my instrument computer, I receive an error message that suggests the camera cannot communicate with the computer. What should I do?

A: The most common reason for loss of communication between the camera and computer is a slightly disconnected camera cable. Please power down both the LightScanner and computer and disconnect and reconnect the camera cable. Make sure the camera cable is not kinked or connected at an off-angle; both which can be prevented by making sure the rear of the LightScanner and computer are at least 6 inches from the wall. If this does not fix the problem, contact Technical Support.

Q: I noticed that a few of my samples contain signals that are far more intense than others, and the intensity for the remaining samples now appears very weak. Is there any way I can fix this?

A: On occasion a few wells may be missed when adding mineral oil, which causes evaporation of the sample. Extreme variation in well fluorescence may cause the camera to use a very short exposure time, which makes all other wells look much weaker. Rehydrate evaporated wells with 10 µL of water and overlay each with 20 µL mineral oil and remelt the plate. Rehydration of evaporated wells should correct the camera exposure and allow remaining samples to be analyzed properly. If excessive signal is still produced from the rehydrated wells; set the camera exposure time so that no wells are magenta in color. Please note that data from rehydrated wells should only be used as a last resort if complete sample analysis of affected samples cannot be repeated. If you do rehydrate samples after PCR, make sure to use good technique to minimize the chance of contaminating yourself with amplicon.

Q: How do I manually set the camera exposure time?

A: In the initial Run screen in LightScanner software, input a value in the Exposure setting in the Run Controls area then click on Fine Tune Map. Repeat the process while adjusting the input value so that the brightest wells on the plate image to the right are red in color: magenta wells represent camera saturation, and the exposure time should be reduced to avoid saturation. Typical exposure times for quality PCR reactions are about 25 to 125 ms. The maximum fluorescent signal at the beginning of a melt should be approximately 1500–1800.

Q: My plate will not eject from my LightScanner. What should I do?

A: If you are removing adhesive film from your plate before inserting it into the LightScanner, it is not uncommon for the residual adhesive to build up inside the LightScanner, which causes the plate to stick inside the instrument and not eject. Please remove all buildup of adhesive inside your LightScanner using a swab and methanol. If you do not suspect that adhesive buildup is the problem, the plate ejector motor may be faulty; please contact Technical Support for assistance. Please note that buildup of adhesive residue can be avoided by using one of the recommend real-time sealing films listed in the plate and sealing film compatibility chart located on Appendix B of your *LightScanner Operator's Manual* (also see http://www.idahotech.com/pdfs/LightScanner/PCRPlateVendors.pdf). The recommended films do not interfere with the fluorescent signal and can be left in place during melting.

Q: My sample results appear to be inverted when compared to how I aliquotted them into my plate. What may be the cause?

A: Results may be inverted when the appropriate plate or plate/adapter combination is not used. Please use the plates listed in Appendix B of your LightScanner Operator's Manual and if half-skirted plates are being used, be sure to use the plate adapter as described in chapter 4 of the

LightScanner Operator's Manual under “Loading a Plate and Starting a Run.” If you are already using the plate adapter or full-skirted plates as recommended and your results are still inverted, please contact Technical Support.

Q: The plate door doesn’t close on my LightScanner when I shut it off. Can this hurt my LightScanner?

A: No. The door closes only when the LightScanner senses that a plate has not been inserted.

Q: What is the lowest volume that can be used in 96-well plates for detection on the LightScanner?

A: We recommend no less than 10 µL with a 20-µL oil overlay. A 10-µL final volume typically produces robust PCR products for detection by the CCD camera.

Q: I have limited template DNA; how can I conserve template DNA without compromising results?

A: The amount of starting template DNA is easy to evaluate experimentally. We recommend performing a dilution series using some samples with known mutations to determine how low you can go and still reliably detect mutations. Conservatively, we would recommend using 2–4 ng of template DNA per reaction.

Q: What are the negative effects of poor plate map calibration?

A: Poor calibration can lead to subtle differences in melt curve shape. When performing the calibration, be sure to click on the geometric center of the wells, not the brightest region of each well. Refer to the “Calibration” section in Chapter 1 of the LightScanner Manual for proper calibration technique.

Q: My melt curves are wavy. Is there something wrong with my LightScanner?

A: A common reason for wavy melt curves is improper sealing film or the presence of air bubbles in sample wells. To avoid this issue, please make sure you are using mineral oil and sealing films that are recommended in Appendix B of the LightScanner Operator’s Manual. Always be sure to centrifuge each plate for approximately 3 min. at 2000 rpm before placing it in the LightScanner.

Q: Can I add mineral oil after PCR instead of before?

A: Addition of mineral oil post-PCR is not recommended. Adding oil after PCR increases the chance of contamination, which can compromise future results. Additionally, pipetting mineral oil into the sample wells before PCR is the best way to prevent evaporation during PCR and Hi-Res Melting, even with the addition of heated lids on thermocyclers. When using 10 µL reactions, evaporation of just 1 µL may cause a 10% change in reaction concentrations and ratios, which may significantly impact PCR efficiency.

Q: Is the effect of bound fluorescence of LCGreen Plus similar to that of other dyes?

A: The fluorescent enhancement of LCGreen Plus upon binding dsDNA is similar to other asymmetric cyanines (ca. 1000-fold), but depends on the temperature. At low temperatures (20–50°C), there is significant binding to ssDNA (e.g., primers), also similar to many asymmetric cyanines.

Q: What is the structure of LCGreen Plus, and how does it bind on a DNA molecule?

A: The exact structure of LCGreen dyes are kept a trade secret, as is the usual practice for commercial dyes in this field. The binding mode of LCGreen dyes has not been investigated, but by analogy to SYBR Green 1, it is likely that both intercalation and surface binding occur, with fluorescence dependent on the latter. Dujols et. al. 2006 (1).

Q: What are the excitation and emission wavelengths of LCGreen Plus?

A: The excitation and emission maxima of LCGreen Plus in the presence of DNA and PCR buffer are 460 nm and 475 nm, respectfully. Herrmann et. al. 2007(2).

High Sensitivity Master Mix

Q: What is the recommended amplicon size range when using the High Sensitivity Master Mix?

A: Sensitivity enhancement when using internal calibrators is dependent on each sequence and the length of each amplicon. As amplicon length increases, sensitivity typically decreases. The recommended range for this master mix is 40–120 base pairs.

Q: Are there Tm restrictions when using the High Sensitivity Master Mix?

A: Yes. The entire sample's melt transition must not overlap calibrator melts. This means that all genotypes of your amplicon must not be melting below 64°C or above 90°C. Please note that the Tm restriction cannot be overcome by the addition of adjuvants because the calibrator melting profiles are affected just the same as those of the amplicon. In general, the Tms or peaks from your products should fall between 70°C and 86°C for success. This can be estimated by using our free software tool and inputting the sequence of the amplicon (click on http://www.idahotech.com/Support/ and go to the bottom of the page under Probe Melting Software). Add ~4°C for best results.

Q: I suspect that the Tm difference between the homozygous alleles I am investigating is greater than 0.5°C. Can I still use the High Sensitivity Master Mix and will it be effective?

A: Yes. Not only does the internal calibrator temperature alignment improve sensitivity of detection of alleles that differ in Tm by less than 0.5°C, it enhances the precision and accuracy of sample grouping, which improves melting profile difference detection.

Q: I am attempting to optimize PCR conditions for a 120-bp product with the new High Sensitivity Master Mix. I used 1 µL DNA at 10 ng/µL. The bands on the gel were too bright, and I cannot see the internal calibrator peaks at 62°C and 92°C in my melt data. Am I doing something wrong?

A: Highly fluorescent PCR products typically lead to reduced camera exposure time, which can cause internal calibrator signals to appear very weak. To help alleviate the issue, reduce PCR product yield by lowering primer concentrations and be sure that no more than 0.25 µM of each primer is contained in the reaction. It may be best to determine optimal primer concentrations by performing a primer titration experiment. Reducing the size of the amplicon will increase the fluorescent signal of the calibrators , which in turn will allow success.

Q: Can the High Sensitivity Master Mix with the internal calibrators be used with amplicons larger than 120 bp?

A: Yes, however, larger PCR products inherently produce greater fluorescent signals when saturating dyes are used, which may hinder detection of the temperature calibrators. As fluorescent signal of the PCR product increases, the relative detection of the internal calibrators may decrease. When using the High Sensitivity Master Mix for larger-than-recommended fragments, we suggest PCR optimization and primer concentration titration such that the lowest primer concentration is used that still generates robust PCR product.

Q: Will the addition of an adjuvant such as DMSO to the High Sensitivity Master Mix reduce the Tm of the lower calibrator relative to the high calibrator?

A: No. The addition of an adjuvant will decrease both calibrator Tm's by the same amount, and the relative Tm difference will remain the same. However, if your PCR product has a very high GC content, adding 5–10 bases of T's and A's to each primer will probably lower the small amplicon Tm by several degrees, allowing success.

LunaProbes

Q: What should the Tm of my LunaProbe be relative to the Tm of my primers?

A: The probe should be designed such that the Tm of the perfect probe or target match is at least 2°C less than the lowest primer Tm. If the most stable probe Tm is too close or higher than that of the primers, the probe may actually inhibit extension due to the exonuclease positive nature of the enzyme used in LightScanner master mixes.

Q: Is there a minimum ΔTm between the perfect match and mismatch when using LunaProbes?

A: Yes, the difference between match and mismatch probes should be greater than 3°C.

Q: How long should my LunaProbe be, and are there any restrictions relative to amplicon length?

A: The probe should be between 20–35 bases and should be at least 10% the length of the amplicon to ensure detection of the probe or target duplex.

Homozygous Mutations

Q: How can we reliably detect homozygous mutations on the LightScanner?

A: Any sample that has a different melting profile as determined by the software from the common or wild-type sample should be classified as a possible mutant. Heterozygous mutations typically produce relatively different melt profiles when compared to their wild-type counterparts; however, homozygous mutations can be a bit more challenging. To ensure you are not missing homozygous mutations, we recommend either sample mixing, the use of High Sensitivity Master Mix for small amplicons, or amplicon-specific sensitivity level optimization (determining how much melt profile variance you typically see between wild-type samples so you know how different a mutant will look) during data analysis.

References

1. Dujols VE, Kusukawa N, McKinney JT, Dobrowolski SF, Wittwer CT. 2006. High-resolution melting analysis for scanning and genotyping. *Real-Time PCR.* Dorak MT (Ed.), Garland Science, New York, 157–171.
2. Herrmann MG, Durtschi JD, Bromley LK, Wittwer CT, Voelkerding KV. Expanded instrument comparison of amplicon DNA melting analysis for mutation scanning and genotyping. *Clin. Chem.* 2007. 53(8):1544–8.

Additional References

For more information on the various techniques used with the LightScanner, please refer to a list of references found on http://www.idahotech.com/Support/References/References.html. There is also a PubMed link at this same location where the full papers can be accessed.