A Cross Platform Comparison of Melting Curve Analysis

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ABSTRACT

INTRODUCTION: Melting analysis of PCR product was first performed on the LightCycler 10 years ago. Now, melting analysis is a standard function on all real-time PCR instruments. Recent advances in DNA melting analysis, including high resolution melting and specialized dyes, have increased the capabilities of melting analysis for genotyping and screening. With the gaining acceptance of melting analysis as a diagnostic tool, there is a need to characterize the ability of commercially available real-time PCR instruments to perform melting analyses. In the current study, a systematic evaluation of melting analysis capability was undertaken employing eight real-time machines from six vendors in resolving SNP genotypes by melting with SYBR Green I and LCGreen Plus.
METHODS: Eight instruments capable of real-time melting curve analysis were compared: ABI's Prism 7000 and 7900HT, Bio-Rad's iCycler iQ, Cepheid's SmartCycler, Corbett Research's Rotor-Gene 3000, Idaho Technology's HR-1 and LightScanner, and Roche's LightCycler. The 110 bp product of each ß-globin genotype (wild type: Hb AA, mutant: Hb SS, heterozygote Hb AS) was amplified using a standard 96-well thermal cycler. The resultant amplicon was pooled by respective genotypes and melted at three different rates upon each melting instrument. The resulting melting curves raw data were analyzed using high-resolution in-house software.
RESULTS: Different instruments and dyes were variably successful in the two major applications of melting analysis: genotyping and scanning. Both SYBR Green I and LC Green Plus adequately resolved homozygous genotypes based on Tm Shifts. However, only LC Green Plus was useful for heterozygote scanning. Scanning was not possible with LC Green Plus on laser-based systems because of wavelength mismatch. As expected, instruments specifically designed for high-resolution melting performed well in both genotyping and scanning.
CONCLUSIONS: Different instruments and dyes vary widely in their ability to genotype and scan amplicons by melting analysis. The sensitivity and specificity of amplicon scanning and genotyping depends strongly on the instruments and dyes used.

INTRODUCTION

Many melting techniques employ fluorescently-labeled oligonucleotide probes to genotype short segments of PCR product, by converting melting curves to negative first derivative melting plots that reveal melting transitions of the probe-target hybrid as peaks. PCR products can also be melted in the presence of DNA binding dyes such as SYBR® Green I that differentiate double-stranded from single-stranded DNA by change in fluorescence intensity. Sequence variants are inferred from changes in the melting transition of the PCR product. Different PCR products generally have different melting temperatures, depending on their GC content, length and sequence. However, temperature resolution is limited and small sequence differences, such as single nucleotide polymorphisms (SNPs) are difficult to identify. Recently, techniques for high-resolution amplicon melting have appeared along with the introduction of a new family of LC Green® dyes (Idaho Technology). Most homozygous sequence changes result in a melting temperature (Tm) shift compared to the wild type. In contrast, heterozygous samples are identified not by product Tm, but by differences in melting curve shape. Different genotypes show unique transitions that are revealed by high-resolution melting and subtractive difference plots of the melting curve. There is growing interest and utilization of melting analysis in clinical diagnosis. However, no study has evaluated the melting performance of commercially available real-time PCR instruments. While melting analysis with labeled probes is well documented on many platforms, a comparative analysis of amplicon genotyping and heterozygote scanning on different platforms is lacking. In the current study, a systematic evaluation of melting analysis capabilities was undertaken employing eight instruments from six vendors. As a model analytical target, the sickle cell mutation was chosen. The sickle cell mutation (Hb S) in the ß-globin gene is an A>T transversion in the second nucleotide of codon 6. A 110-bp PCR product including this mutation with a predicted Tm difference between homozygotes (AA and TT) of 0.09°C provides a stringent test for differentiation.

METHODS

INSTRUMENTATION
- Eight instruments capable of melting analysis were available for comparison in our laboratory representing six vendors. Prism® 7000 SDS & 7900HT (Applied Biosystems), iCycler iQ (Bio-Rad), SmartCycler® II (Cepheid), Rotor-GeneTM 3000 (Corbett Research), LightScanner® & HR-1™ (Idaho Technology) and the LightCycler® (Roche).
- All instruments, except the HR-1 and the LightScanner, also provide temperature cycling for PCR.
- New excitation (438/24) and emission (485/20) filters (center wavelength/band pass at half height, (Semrock)) for LCGreen Plus were installed in the iCycler.

PCR TARGET
- Target: Exon one of the ß-globin gene (Genbank: U01317).
- Primers: PCO3 (position: 62150) and PCO4 (position: 62259) (Saiki)
- Sample: DNA was extracted from EDTA-anticoagulated whole blood samples using QIAamp DNA Blood Mini kit (Qiagen, Valencia CA). Samples were handled according to a global ARUP protocol under IRB #7275.
- Genotypes: Validated by sequencing for the ß-globin consensus sequence except at the base resulting in the Hb S mutation. A single patient sample of each genotype was used; wild type, homozygous mutant and heterozygous mutant. Nearest-neighbor thermodynamic parameters predicted Tms of 85.80°C for the wild type and 85.89°C for the homozygous mutant.

AMPLIFICATION
- Instrument: GeneAmp 9700 (Applied Biosystems)
- Cycling Conditions: Holds 30°C for 10 min and 95°C for 10 min,
  35 Cycles @ 95°C for 15 sec, 65°C for 20 sec, and 72°C for 1 sec
  7 min hold at 72°C with subsequent rapid cooling to 4°C.
- Reaction: each well contained 50 µL of 50 mM Tris, pH 8.5 (25°C), 3 mM MgCl2, 0.5 mM each primer, 250 µg/mL bovine serum albumin, 0.2 mM dATP, dGTP and dCTP, 0.6 mM dUTP, 1 U heat-labile Uracil DNA glycosylase, 0.04 U/µL Taq polymerase, 250 ng human genomic DNA containing either a 1:30K dilution SYBR Green I or 1X LCGreen Plus.

MELTING ACQUISITION

Each instrument was run at the manufacturer's recommended sample volume, varying from 10-25 µl. Triplicate samples for each of the three genotypes for each dye were prepared for each instrument (3 x 3 x 2 x 8) and stored in the dark at 4°C prior to melting (< 8hr). Samples positions in instruments with 96-well heat blocks were randomly assigned; the residual wells were filled with water. For plates used in determining temperature uniformity of the heat blocks all 96 wells were filled with wild type PCR product.

After an initial hold at 60°C for 30 sec, the samples were melted by increasing the temperature to 95°C at 0.1°C/sec. Exceptions: ABI 7900 had a mandatory pre-melting cycle of 95°C for 15 sec followed by 60°C for 15 sec, and the ABI 7000, LightScanner and HR-1 had no temperature hold prior to melting. The instruments had different methods of acquiring fluorescence during melting. One method was "continuous" where the melting rate is held constant and the fluorescence is acquired as fast as possible. In contrast, some instruments employed a "step" mode where the instrument acquires fluorescence in fixed temperature steps, ensuring a constant number of data points per °C but at a much slower temperature ramp rate. The iCycler and Rotor-Gene both utilized 10 sec holds at each step for temperature equilibration prior to fluorescence acquisition. On the ABI 7900HT, the melting rate was expressed as, "% heating rate", and was run at the company suggested 2%. On the ABI 7000, only one preset melting protocol was available. On the LightCycler, both continuous and step modes were available. Preliminary experiments showed that although step mode provided more data points, the continuous mode was preferable because of less noise.

MELTING ANALYSIS

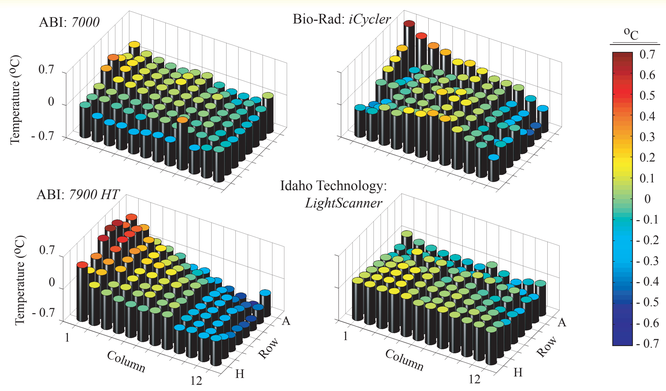
RESULTS:

Thermal Block Melting Profiles

The dynamic thermal uniformity of the heat blocks based on amplicon Tm is shown below.

The LightScanner had the least amount of thermal variation with 0.35°C across the plate. The heat blocks varied in thermal uniformity with ABI 7900 having 1.24°C thermal variation, the ABI 7000 with 0.66°C and Bio-Rad's iCycler with 1.10 °C.

Heat block systems with greater thermal control had lower Tm standard deviations.

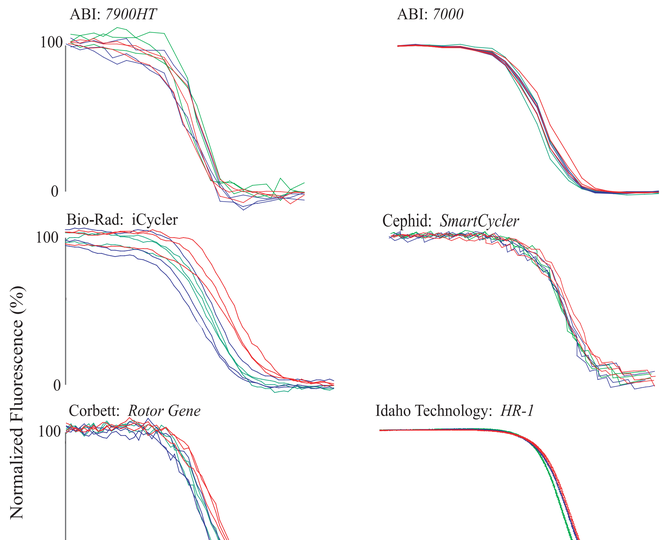


Melting Effects of SYBR Green I

The mean +/- standard deviation of the Tm across all instruments was 85.40 +/- 0.49°C for the AA and 85.49 +/- 0.51°C for the TT genotypes and are only 0.4°C off from nearest neighbor predicted values. The Tm difference between homozygous genotypes (TT - AA) across all instruments was 0.09 +/- 0.17°C. This mean is exactly predicted from nearest neighbor parameters, although the magnitude of the standard deviation suggests that there will be difficulty genotyping individual samples.

In general, variation between samples of the same genotype precludes accurate genotyping. An exception may be the HR-1 instrument where the AA genotype appears to the left of the AT and TT genotypes.

On all instruments, the heterozygous AT genotype is not distinct from the homozygous genotypes when SYBR Green I is used.



Effects of Melting with LC Green Plus

The mean +/- standard deviation of the Tm across all instruments using LCGreen Plus was 85.43 +/- 0.42°C for the AA and 85.62 +/- 0.50°C for the TT genotypes with a Tm difference (TT - AA) of 0.19 +/- 0.15°C.

Normalized melting curves using LC Green Plus on all instruments compatible with the dye. With only three samples per genotype, homozygotes appear distinguishable on the ABI 7000, the SmartCycler, the Rotor-Gene and the HR-1. However, the amount of variation on some instruments may preclude accurate genotyping with a larger sample size. When the melting curves were temperature-shifted heterozygotes could be identified on all instruments.

The eight different instruments varied by an order of magnitude in the standard deviation of replicate samples. The standard deviations of the four 96-well plate instruments (0.092 to 0.173°C) were greater than instruments based on circulating air (0.045°C) or individually controlled samples (0.018 and 0.065°C). For heterozygote detection, temperature-shifting reduces the variation within genotypes, resulting in Tm standard deviations for scanning from 0.012 to 0.065°C.

Because homozygous melting curves only differ in position (Tm) and not shape, the ability of each instrument to distinguish homozygous genotypes depends only on the standard deviation of the measured Tm and the actual Tm difference between genotypes. The estimated error rates (assuming normal distributions) for the seven instruments at Tm differences of 1.0, >0.5, 0.25, and 0.09°C are shown.

Melting Curves for the ABI 7900 with LC Green Plus are unavailable due to the instruments fluorescence excitation and emmission incompatibility with the dye.

A. Normalized Melting Curves B. Temperature Shifted Curves

ABI: *7000*
Sample Number: 96
Sample Volume: 25ul
Thermal control: Peltier
Acquisition mode: Continuous
Data Points /°C : 3.1
Melting Rate : 0.022 °C/s

Bio-Rad: *iCycler*
Sample Number: 96
Sample Volume: 25ul
Thermal control: Peltier
Acquisition mode: Step
Data Points /°C : 10
Melting Rate : 0.009 °C/s

Cephid: *SmartCycler*
Sample Number: 16 / 1
Sample Volume: 25ul
Thermal control:
Thermoelectric/ Air
Acquisition mode: Continuous
Data Points /°C : 10
Melting Rate : 0.1 °C/s

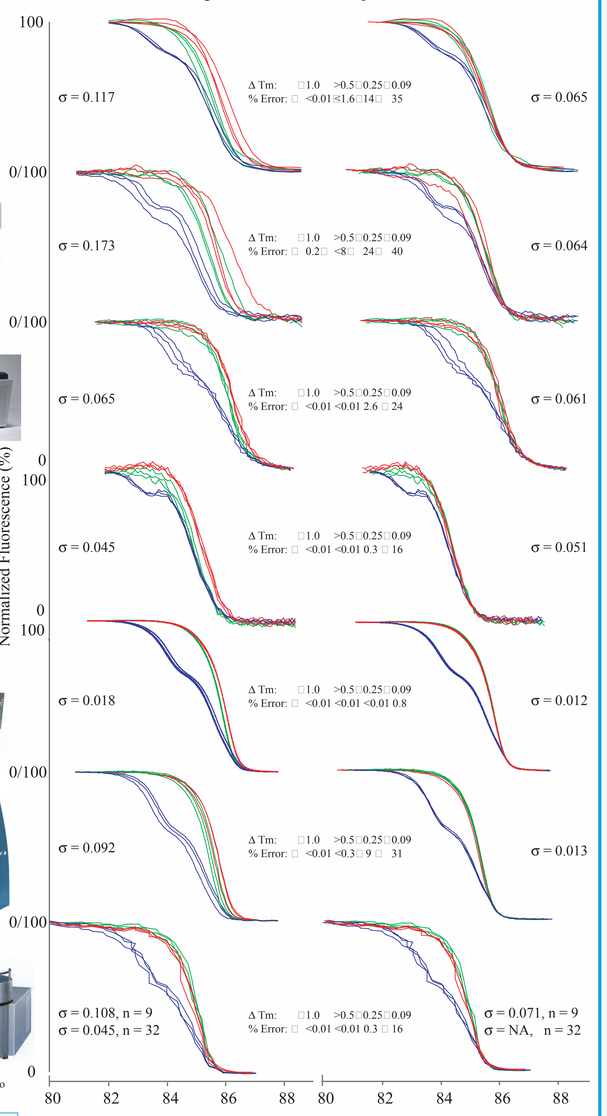
Corbett: *Rotor-Gene*
Sample Number: 72
Sample Volume: 20ul
Thermal control: air
Acquisition mode: Step
Data Points /°C : 10
Melting Rate :
0.008 °C/s

Idaho Technology: *HR-1*
Sample Number: 1
Sample Volume: 10ul
Thermal control: Thermoelectric
Acquisition mode: Continuous
Data Points /°C : 200
Melting Rate : 0.1 °C/s

Idaho Technology: *LightScanner*
Sample Number: 96/364
Sample Volume: 10ul
Thermal control: Thermoelectric/Air
Acquisition mode: Continuous
Data Points /°C : 14
Melting Rate : 0.1 °C/s

Roche: *LightCycler*
Sample Number: 32
Sample Volume: 10ul
Thermal control: Air
Acquisition mode: Step/Continuous
Data Points /°C : 14 @ n=9
1.2 @ n=32
Melting Rate : 0.1 °C/s

LightCycler data shown is for n=9
σ values for n=32 could not be calculated due to too few data points.



Wild Type, Heterozygote, Mutant

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