LightScanner™ Hi-Res Melting™ Comparison of Six Master Mixes for Scanning and Small Amplicon and LunaProbes™ Genotyping

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Introduction

Commercial master mixes are convenient and cost-effective solutions for producing reliable scanning and genotyping results. BioFire Defense was the first to offer Hi-Res Melting™ instruments, analysis software, and chemistry solutions. This study performed side-by-side comparisons of six commercial master mixes indicated for Hi-Res Melting:

- LightScanner Master Mix (Figures 1, 3) and the LightScanner High-Sensitivity Master Mix (Figure 2) (with calibrators) with LCGreen® Plus dye (BioFire Defense, LLC)
- LightCycler® 480 High Resolution Melting Master with ResoLight dye (sold by the Roche Group; LightCycler is a registered trademark of the Roche Group)
- SsoFast EvaGreen® Supermix with EvaGreen dye (sold by Bio-Rad Laboratories; EvaGreen is a registered trademark of Biotium, Inc.)
- Fast EvaGreen® qPCR Master Mix for qPCR and high-resolution melting with EvaGreen dye (sold by Biotium, Inc.; EvaGreen is a registered trademark of Biotium, Inc.)
- EXPRESS SYBR® GreenER™ qPCR SuperMix Universal (sold by Invitrogen; SYBR is a registered trademark of Molecular Probes Inc.; GreenER is a trademark of Life Technologies, Inc.)
- MeltDoctor HRM Master Mix (sold by Applied Biosystems, Inc.)

A panel of human genomic DNA samples with known genotypes in the LIPC (large amplicon scanning), human tyrosine hydroxylase (small amplicon genotyping), and BX647987 (LunaProbes genotyping) genes were used in this evaluation.

Methods

Whole blood was drawn from 30 human donors, and DNA was isolated using standard extraction kits and procedures. Samples were diluted in TE 1X buffer, pH 8.0, to a final concentration of 15 ng/µL. Genotypes for all comparisons were identified using Hi-Res Melting technology and sequence-verified. All master mixes were used in accordance with manufacturer recommendations. We optimized PCR protocols for each master mix to generate the cleanest amplicons for analysis on the LightScanner system. Standard agarose gels were run to verify annealing temperature optimization. PCR duplicates (Figure 1) or triplicates (Figures 2 and 3) were run for each DNA sample. All PCRs for master mix comparisons were performed on calibrated 96-well plate thermal block cyclers, followed by Hi-Res Melting on the LightScanner. Data analysis was performed using standard software supplied with the LightScanner.

High Sensitivity master mix (with calibrators) was used in place of the LightScanner master mix in the small amplicon genotyping study. It should be noted that this is the only commercial master mix that contains a finely tuned internal calibration system designed to work with High-Res Melting calibration software. Because of this, peak alignment is highest in Figure 2 with this master mix. Using this master mix ensured the highest resolution available despite the inevitable yet subtle sample-to-sample temperature, volume, and buffer variation.

Results

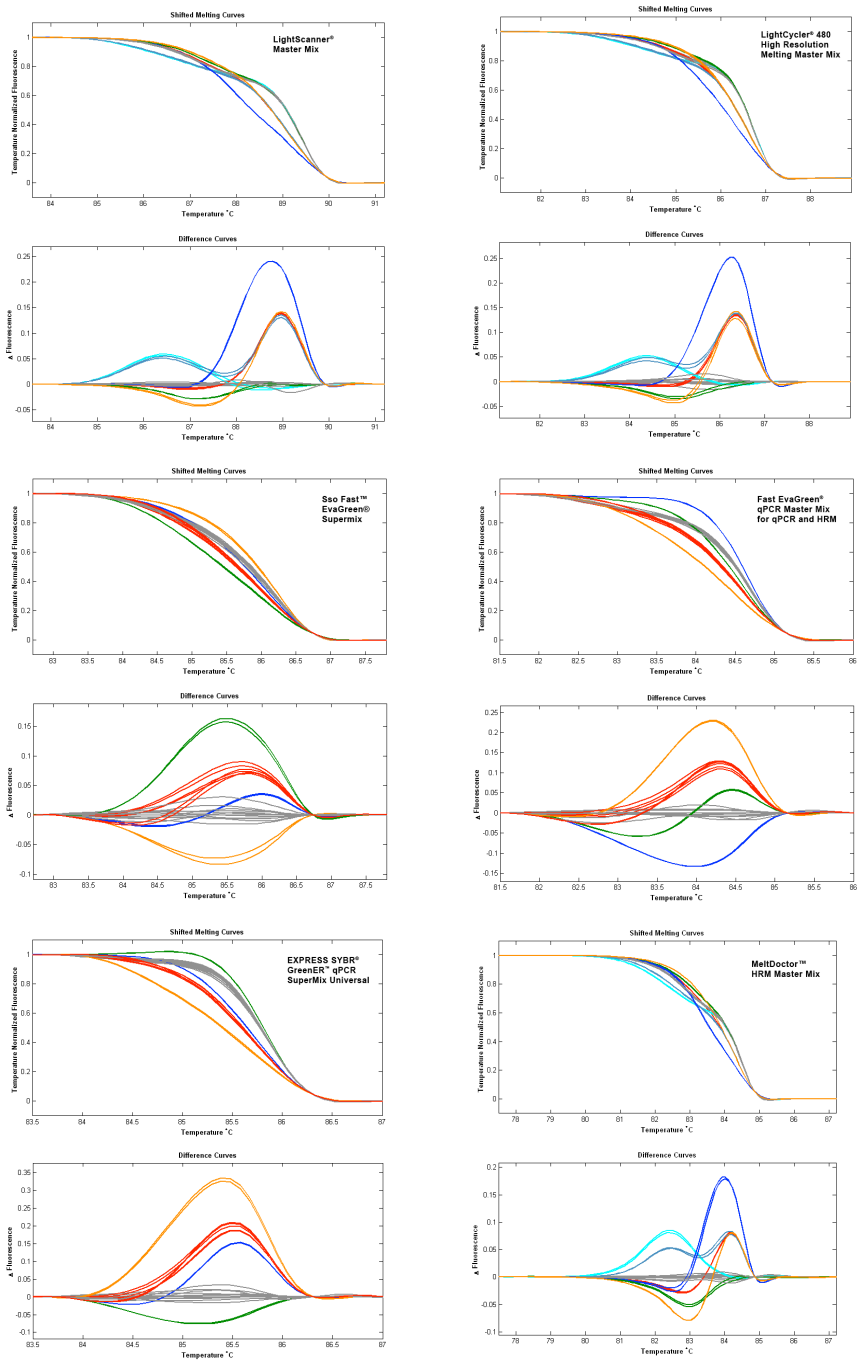

Figure 1. Scanning sensitivity variation across the six master mixes.

Gene scanning results for the LIPC amplicon (302 bp, 52% GC) are shown in Figure 1. LightScanner, LightCycler 480, and MeltDoctor mix correctly identified all seven genotype groups. In contrast, SsoFast EvaGreen, Fast EvaGreen, and EXPRESS SYBR GreenER master mixes were only able to differentiate five groups. This was due to the homozygous variant samples being incorrectly grouped with the wild-type profiles and two different heterozygous variants being grouped together. This decreased sensitivity is probably caused from reduced or absent heteroduplex content at approximately 87.5oC. This second melting domain is clearly observed with the LightScanner Master Mix and is partially observed with the LightCycler 480 mix.

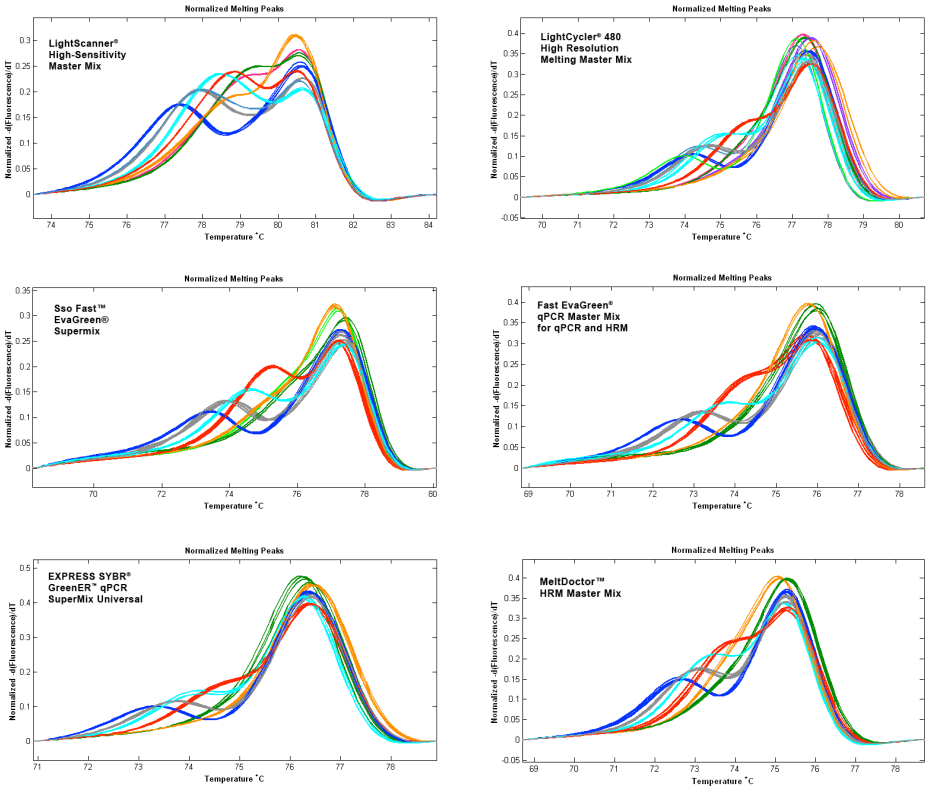

Figure 2. Human tyrosine hydroxylase locus small amplicon genotyping results.

The human tyrosine hydroxylase locus was assayed using a set of primers immediately flanking a common short tandem repeat used in forensics (HUMTHO1) yielding a variable length fragment ranging from 67–82 bp. Results are shown in Figure 2 above. Sample genotypes were established by fragment analysis performed independently by the Los Angeles Police Department's forensics laboratory.

Regular LightScanner Master Mix did not perform as well (data not shown) as the LightScanner High Sensitivity Master Mix. The regular LightScanner Master Mix is designed for scanning—not small amplicon genotyping, which requires temperature calibrators for the highest Tm separation.

The High Sensitivity Master Mix correctly identified all eight genotype combinations. The MeltDoctor, SsoFast EvaGreen, Fast EvaGreen, and EXPRESS SYBR GreenER master mixes identified six of eight groups correctly. Several errors were found with the LightCycler 480 mix. In two instances, two genotypes were combined into a single group and also resulted in three unique genotypes being clustered into only two groups. The LCGreen dye in the High Sensitivity Master Mix creates maximal heteroduplex content as can be seen in Figure 2 (note green and orange group profile differences). All other dyes have less heteroduplex content, partly explaining the loss of sensitivity in this small amplicon genotyping application.

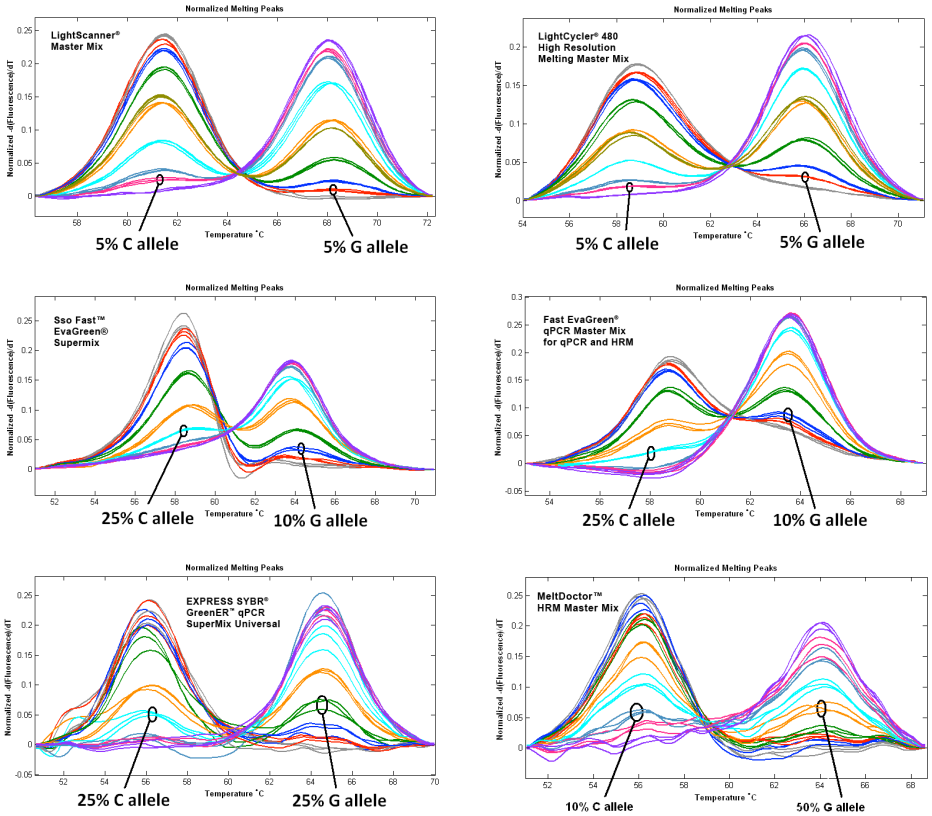

Figure 3. Sample mixing to demonstrate LunaProbes sensitivity.

LunaProbes genotyping results for the BX647987 locus are presented in Figure 3 above. A common G:C single nucleotide polymorphism (SNP) (rs1869458) was assayed in the human BX647987 gene using a LunaProbes assay to evaluate each product's ability to detect minimal amounts of a given allele. In this case, the probe was designed as a perfect match to the G allele while creating a single base pair mismatch to the C allele. A homozygous G and homozygous C sample were identified, quantified, and mixed to create fractions of both alleles down to 5% in a 95% background of the other allele. A natural heterozygous sample was also run for comparison and validation of the mixed samples. Using the LightScanner and LightCycler 480 master mixes, the 50%C:50%G mixed sample (orange) performs similarly to the true heterozygous sample (olive green) relative to allele peak heights, verifying the mixed samples were accurately diluted.

Table 1. Detection Sensitivity of rs1869458 LunaProbes for all high-resolution melting master mix products.

Supplier	High-resolution Melting Master Mix	Dye	C allele Sensitivity	G allele Sensitivity	Primer asymmetry
BioFire Defense	LightScanner™ Master Mix	LCGreen Plus	5%	5%	5:1
Roche	LightCycler® 480 High Resolution Melting Master	ResoLight	5%	5%	10:1
Bio-Rad	SsoFast™ EvaGreen® Supermix	Evagreen	25%	10%	5:1
Biotium	Fast EvaGreen® qPCR Master Mix for qPCR and HRM	Evagreen	25%	10%	5:1
Invitrogen	EXPRESS SYBR® GreenER™ qPCR SuperMix Universal	SYBR GreenER	25%	25%	10:1
ABI	MeltDoctor™ HRM Master Mix	SYTO 9	10%	50%	10:1

Conclusions

These experiments show that although several master mix products are marketed for high-resolution melting applications, the LightScanner Master Mix and High Sensitivity Master Mix with LCGreen Plus dye provide the best sensitivity and support the broadest range of applications for high-resolution melting. Experiments for each master mix product were independently optimized to produce high-quality PCR amplicons. LightScanner Master Mix with LCGreen Plus dye yielded the best overall results. This is presumably due to its ability to saturate PCR products and yield maximal heteroduplex detection relative to other master mixes. The other commercial master mix products tended to identify lower heteroduplex percentages in the melting curves resulting in reduced melting domain differentiation. Ultimately, this trend leads to a reduction in mutation scanning sensitivity. Small amplicon genotyping results clearly show increased heteroduplex content as well as tighter peak alignment. This enhancement over the other master mixes results from the unique calibration system included in the High Sensitivity Master Mix and associated LightScanner instrument and software. In addition, greater primer asymmetry was required for adequate LunaProbes signal in several master mixes (Table 1). A higher primer asymmetry makes PCR more difficult and reduces efficiency. For optimal high-resolution melting, a master mix and dye that provide greater LunaProbe signal at a given primer asymmetry is preferred, all else being equal.

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BioFire Defense, LLC formally known as Idaho Technology, Inc.

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