Comparison of Five PCR Master Mix Products for Hi-Res Melting® with 4 Different dsDNA Binding Dyes

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INTRODUCTION

Commercial master mix products are a convenient, cost effective solution for Hi-Res Melting™ applications. The fi rst commercial master mix to include a dsDNA saturation dye for Hi-Res Melting was the LightScanner® Master mix with LCGreen® PLUS dye (Idaho Technology, Inc.). Recently, products using EvaGreen® (Biotium and BioRad), SYBR® GreenER™ (Invitrogen), and LightCycler® 480 ResoLight (Roche) have appeared on the market. The purpose of this study was to investigate the utility of these products for Hi-Res Melting applications. Hi-Res Melting applications included large amplicon gene scanning for heteroduplex detection, small amplicon genotyping, and LunaProbe™ genotyping.

METHODS

To evaluate these products, genomic targets with known SNPs in the LIPC (scanning), CPS1 (small amplicon), OTC (LunaProbe), ADH4 (LunaProbe), human tyrosine hydroxylase (small amplicon), and HFE (multiplexed LunaProbes) genes were used. The CPS1 small amplicon and OTC LunaProbe SNPs were base-neutral A:T changes with nearest neighbor base symmetry. The homozygous forms of these SNPs represent the greatest genotyping challenge due to extremely small ΔTms between homozygous genotypes.

Assays were independently optimized with each master mix product with PCR product specificity confirmed by Hi-Res Melting and agarose gel analysis.

FIGURE 1.

Gene Scanning
LIPC, 5 SNPs, 302 bp

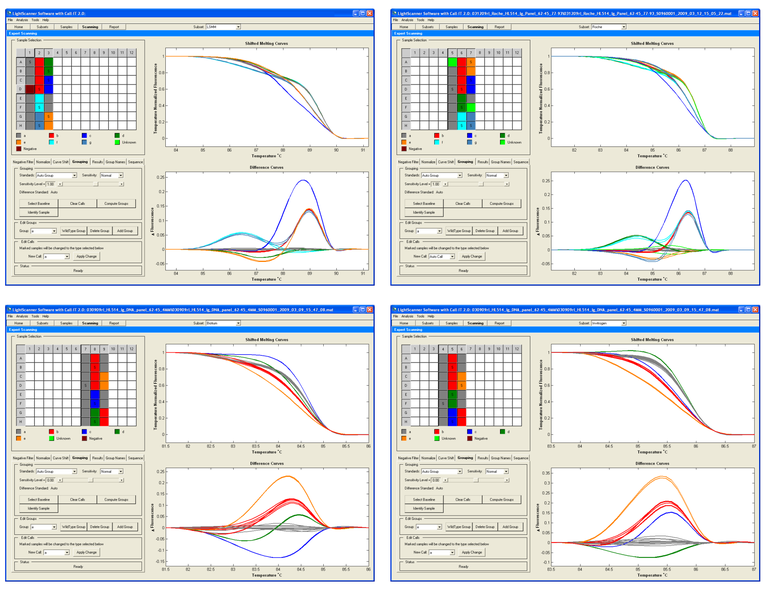


FIGURE 2.

LunaProbe Genotyping
OTC, A:T SNP, 188 bp

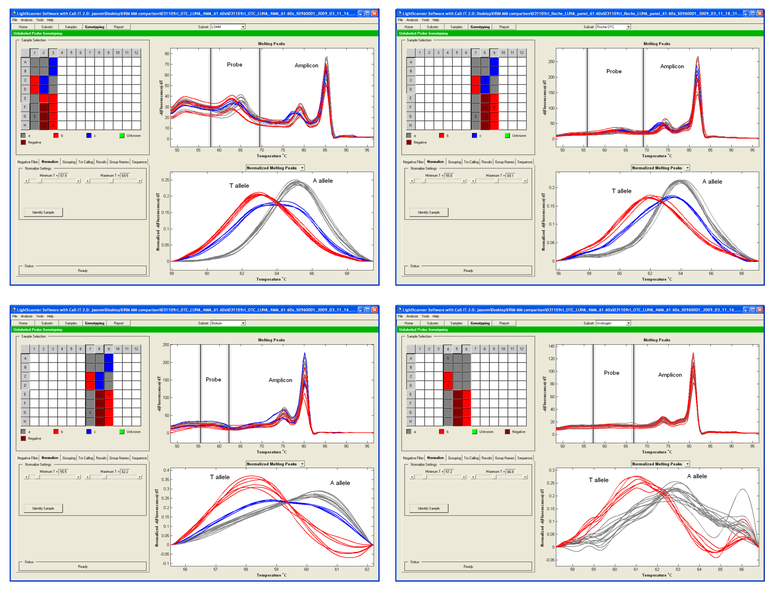


FIGURE 3.

Small Amp Genotyping
CPS1, A:T SNP, 51 bp

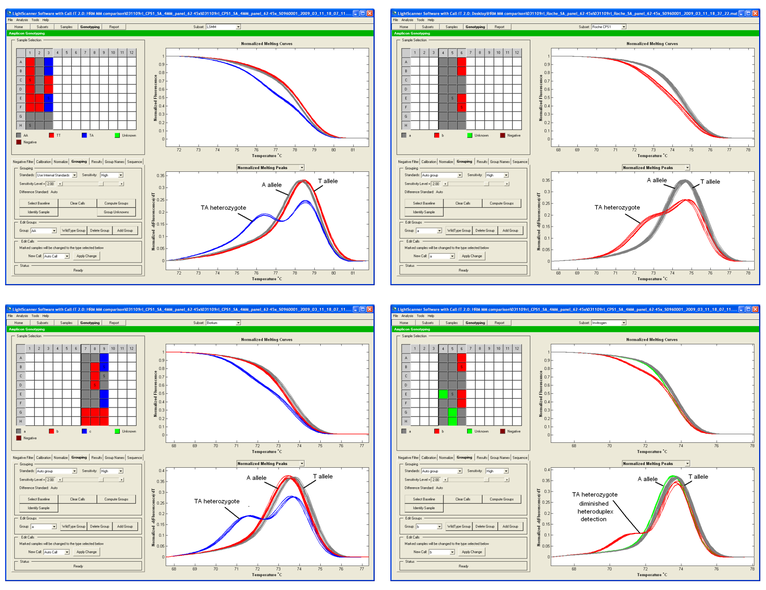


FIGURE 4.

Small Amp Genotyping
HUM *THO1* STR, 67-82 bp

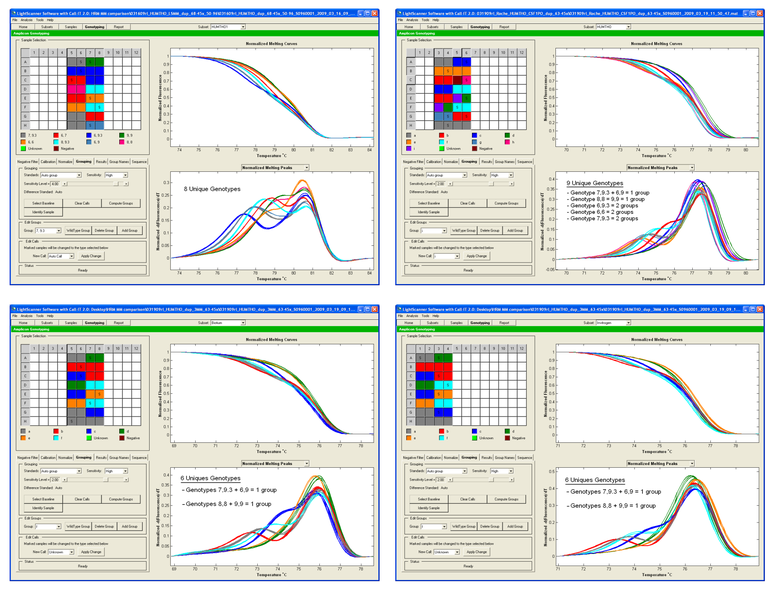


FIGURE 5.

LunaProbe Genotyping
Allele Fraction Sensitivity
ADH4, C:T SNP, 108 bp

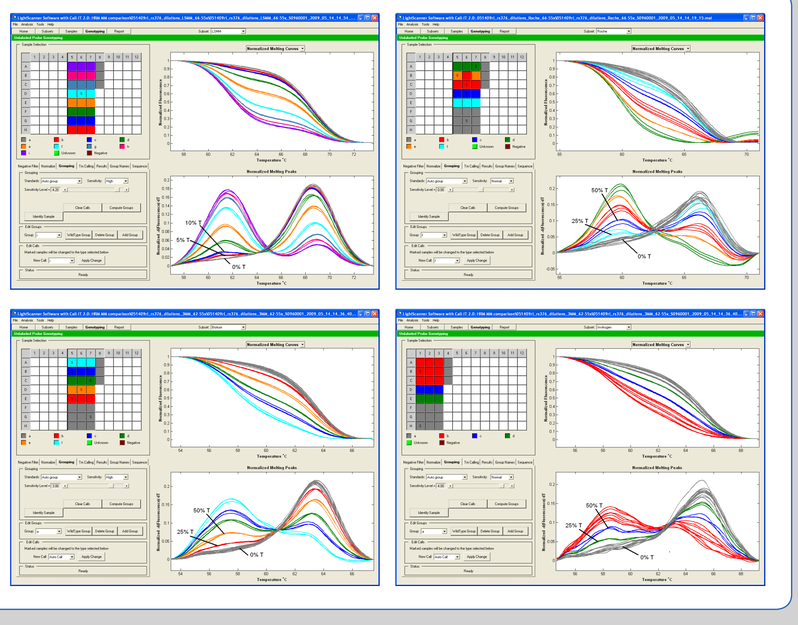


FIGURE 6.

LunaProbe Genotyping
HFE multiplex
C282Y - 87 bp; H63D - 111 bp

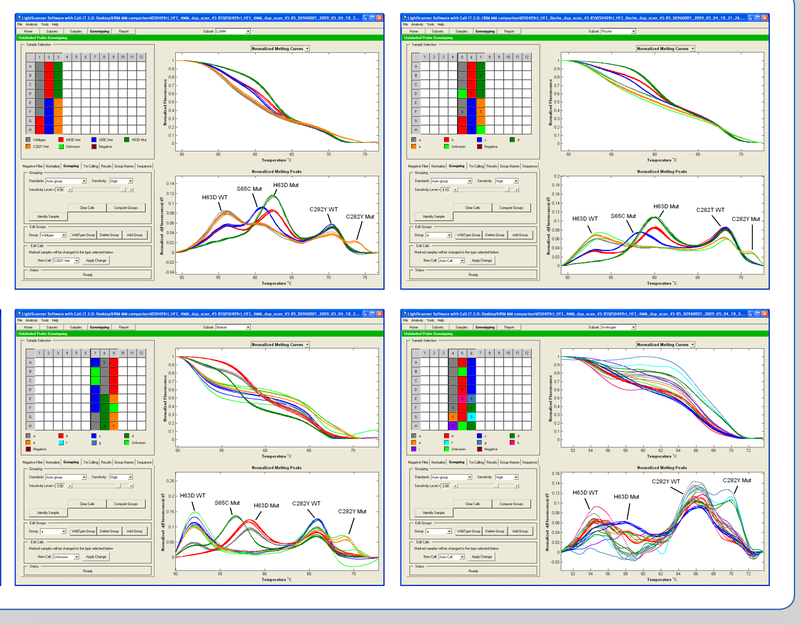
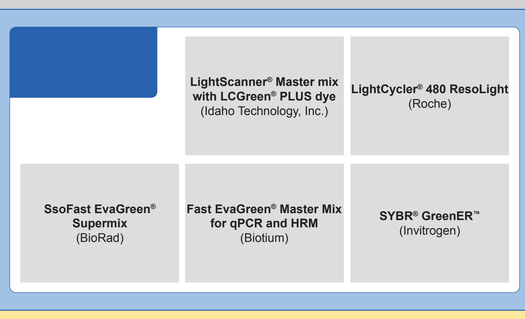


FIGURE KEY

Use this figure key to identify the specific master mix results for the individual in figures 1-6.



RESULTS AND CONCLUSION

Gene scanning results for the large amplicon (302 bp) are shown in **Figure 1**. LCGreen Plus and ResoLight master mix correctly identified all 7 genotype groups, while the EvaGreen and the SybrGreenER mixes were able to distinguish only 5 groups. A homozygous variant was incorrectly grouped with the wildtype group, and two different heterozygous variants were grouped together with EvaGreen and SybrGreenER.

LunaProbe genotyping results for the OTC base-neutral A:T SNP are shown in **Figure 2**. All master mixes except for SybrGreenER were able to correctly genotype the samples. The ResoLight master mix required a greater primer asymmetry (10:1 vs 5:1 for the other products) to generate sufficient probe signal for correct genotyping. Inspection of the melting profiles shows the greatest probe:amplicon signal ratio was generated by the LCGreen Plus mix, with the rest of the products showing minimal probe signal (note probe:amplicon signal ratio in Figure 2).

Small amplicon genotyping results for the CPS1 base-neutral A:T SNP are shown in **Figure 3**. All products correctly identified the heterozygous samples. The LCGreen Plus mix showed the greatest amount of heteroduplex detection (evaluated by derivative peak height and separation), along with the two EvaGreen products. ResoLight and SybrGreenER showed significantly less heteroduplex detection. LCGreen Plus and the BioRad EvaGreen products were the only products that correctly genotyped the homozygous samples.

The human tyrosine hydroxylase locus was evaluated using a set of primers that immediately flank the common STR (HUM *THO1*), yielding a fragment of variable length, dependent on genotype, ranging from 67-82 bp. Results are shown in **Figure 4**. Sixteen samples were genotyped using standard fragment analysis for use in this experiment. The LCGreen Plus master mix correctly identified all 8 genotype combinations. The EvaGreen and SybrGreenER products identified 6 of 8 groups correctly (see figure panels for unique genotypes that were grouped together). The ResoLight mix also put 2 genotypes into a single group in two instances, but also caused 3 different genotypes to each be broken out into 2 groups, for a total of 9 unique groups. One determining factor for success in this assay is likely related to the dyes' ability to distinguish maximal amounts of heteroduplex molecules. LCGreen Plus melt profiles clearly show heteroduplex formation in each genotype identified in this assay, whereas the other dyes tend to show much less heteroduplex formation in most profiles, and virtually no heteroduplex identification in the incorrectly grouped samples.

A common C:T SNP was assayed in the ADH4 gene using a LunaProbe approach in order to evaluate each products ability to detect minimal amounts of a given allele. In this case, the T allele was selected to represent the "mutant", and the probe was designed to create a single C:A mismatch when the T allele was present. A homozygous C and homozygous T sample were quantified and mixed to create fractions of the T allele down to 5%.

LunaProbe genotyping results for the ADH4 locus are presented in **Figure 5.** The SybrGreenER, EvaGreen (Biotium), and ResoLight dyes were able to distinguish down to only 25% of the T allele in a 75% C allele background. The EvaGreen (BioRad) product fared better and was able to detect 10% of the T allele in a 90% C allele background. Only the LCGreen Plus dye was able to detect the 5% T allele fraction.

The well-known HFE loci H63D, S65C, and C282Y were selected for assessment using a multiplexed LunaProbe approach. The results are shown in **Figure 6**. LCGreen Plus mix correctly genotyped all samples and showed very good reproducibility between replicates of each genotype. The EvaGreen and ResoLight mixes identified all of the genotype combinations between the two LunaProbes, with only minor inconsistencies in reproducibility between replicates within genotype. The SybrGreenER mix was unable to produce any clear genotypes due to virtually no probe signal, even though a 10:1 primer asymmetry was used to maximize probe target strand availability.

Non-LCGreen PLUS master mix products consistently displayed a relative decrease in heteroduplex melting domain identification, indicative of a non-saturating dye concentration or a larger dye molecule size, or both, resulting in less fluorescent signal per base pair. All products were able to produce high-quality PCR amplicons; however, Hi-Res Melting applications using small amplicons and LunaProbes were most successful with the LCGreen PLUS master mix, presumably due to its ability to saturate PCR products and yield maximal relative fluorescent signal over a given base pair size range.