Mutant Allele Amplification Bias (MAAB) using Rapid Cycle-Real Time PCR and Hi-Res Melting® with LunaProbes™ on the LightScanner 32™

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INTRODUCTION

Hi-Res Melting® was introduced as a homogeneous method of scanning PCR amplicons for heterozygous sequence variants. Based on the use of dsDNA saturating dyes, high resolution melting is capable of detecting SNP's and In/Del's in amplicons up to 400 bp at a sensitivity >98%. Since its introduction in 2003, additional applications for Hi-Res Melting have been developed, including genotyping using small amplicons or unlabeled probes (LunaProbes™). LunaProbes are 3'-bloc ked to prevent extension during PCR and use LCGreen® Plus to determine genotype based on probe melting temperature (Tm). The probe sequence can be designed to match either allele and is based on maximizing the ΔTm between the perfect match and mismatched probe. LunaProbes can be used for allele quantitation in genotyping, and we have repeatedly detected mutant allele fractions ≤10%. To further enhance LunaProbe sensitivity we developed MAAB methods to enrich the mutant allele during PCR.

MAAB MUTANT ALLELE AMPLIFICATION BIAS

In an effort to bias amplification of the mutant allele relative to the wildtype allele, the LunaProbe is used to retard amplification of the wildtype allele during PCR. The probe is designed to perfectly match the wildtype allele and contain a single base mismatch with the mutant allele. PCR annealing temperature is set such that it is less than the probe Tm of the wildtype allele but ≥ the probe Tm of the mutant allele. By setting this intermediate annealing temperature, the probe will be fully hybridized to the wildtype allele (Figure 1a) but not to the mutant allele (Figure 1b) during PCR, thus blocking or retarding amplification of the wildtype allele relative to the mutant allele.

Figure 1a

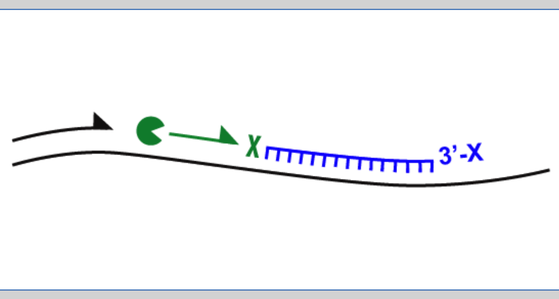
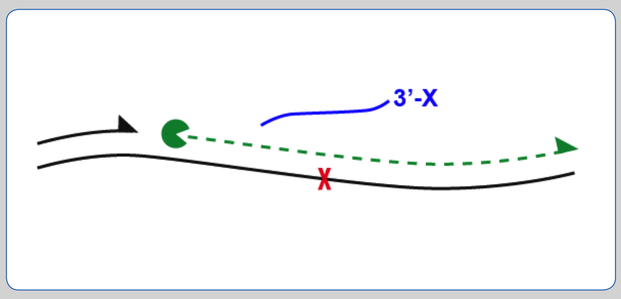


Figure 1b



METHODS

We investigated the use of LunaProbes to discriminate the mutant allele at ≤1% relative to the wildtype allele. We examined the range of annealing temperatures necessary to preferentially amplify the probe mismatch allele (mutant) in the presence of both exonuclease positive and negative enzymes. We chose targets relevant to the application of this technique: human TP53 exon 8 bearing cancer mutations; human PAH exon 11 bearing PKU mutations; and a gene target in malaria where mutations cause resistance to standard drug therapy. An annealing temperature gradient experiment on a standard thermal block cycler was used to determine the LunaProbe Tms and whether MAAB could be induced in these targets.

RESULTS

The exonuclease positive polymerase's (NEB and Roche FastStart) exhibited no MAAB across a relevant range (65-75ºC) of annealing temperatures (Figure 2).

Detection sensitivity of the mutant allele without MAAB was determined to be 5-10% for each target using the LunaProbe (Figure 3). TP53 x8 and PAH x11 both showed characteristics of mutant allele enrichment across the annealing temperature gradient on the thermal block (Figure 4). The parasite target showed no enrichment of the mutant allele using the thermal block protocol (Figure 5). We then tested a range of annealing temperatures using rapid cycle real time PCR followed by high resolution melting on the LS32™ instrument (Figure 6). Mutant allele enrichment was enhanced in TP53 x8 and PAH x11 relative to the thermal block. The parasite target displayed a significant MAAB effect using rapid cycle PCR (Figure 7) using an annealing temperature of 56ºC.

Further mixing of the homozygous WT and Mutant allele parasite samples demonstrated that detection of the mutant allele down to <1% was possible (Figure 8). Analysis of the qPCR data for the 100% WT and Mutant samples confirmed the Mutant allele enrichment was approximately 10X due to MAAB (Figure 9). These results are in agreement with the increase in detection sensitivity of the LunaProbe using MAAB compared to the sensitivity without MAAB applied.

Figure 2

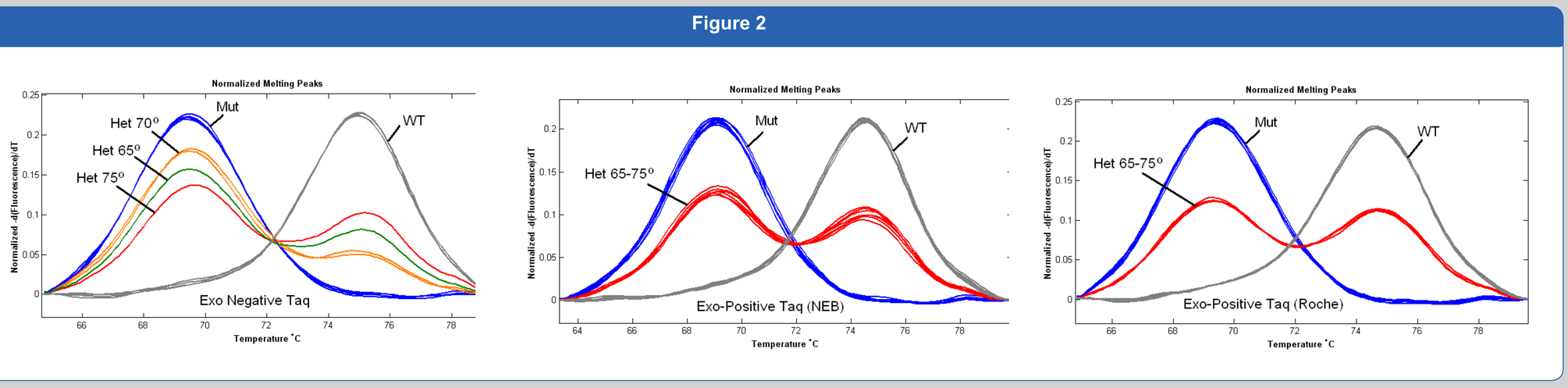


Figure 3

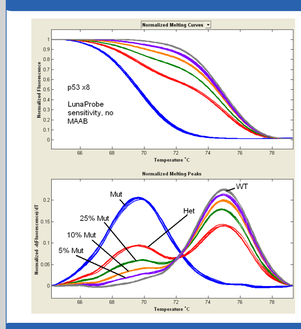


Figure 4

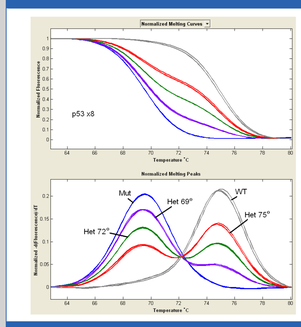


Figure 5

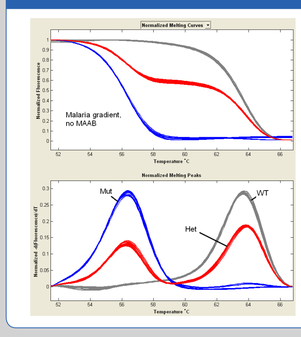
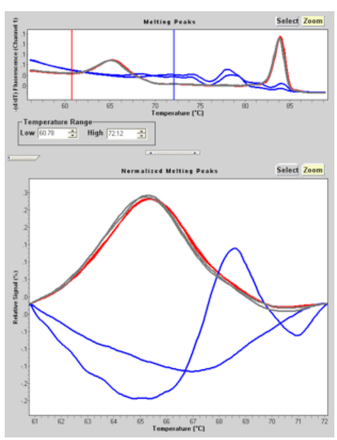


Figure 6

60C ann



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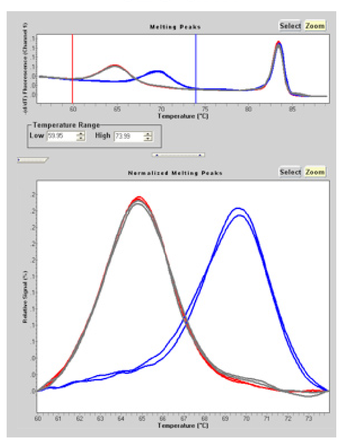

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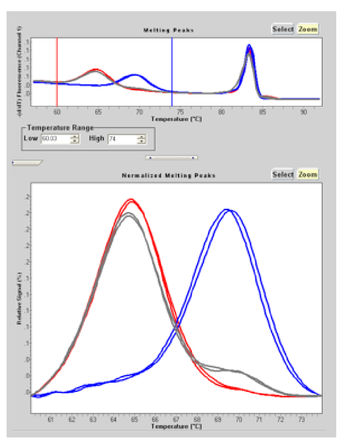
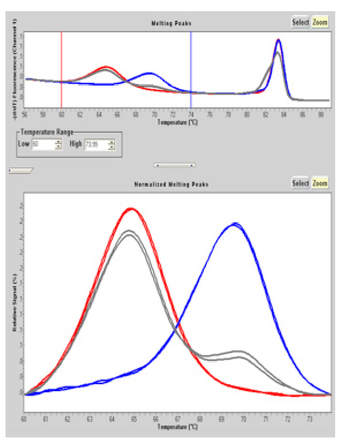
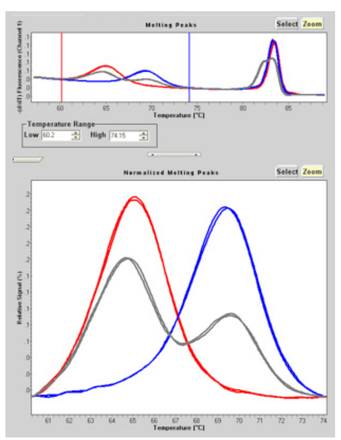


65C ann



68C ann



70C ann

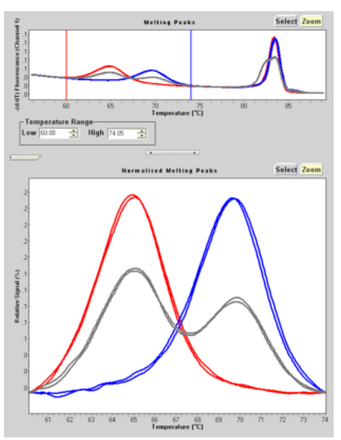


Figure 7

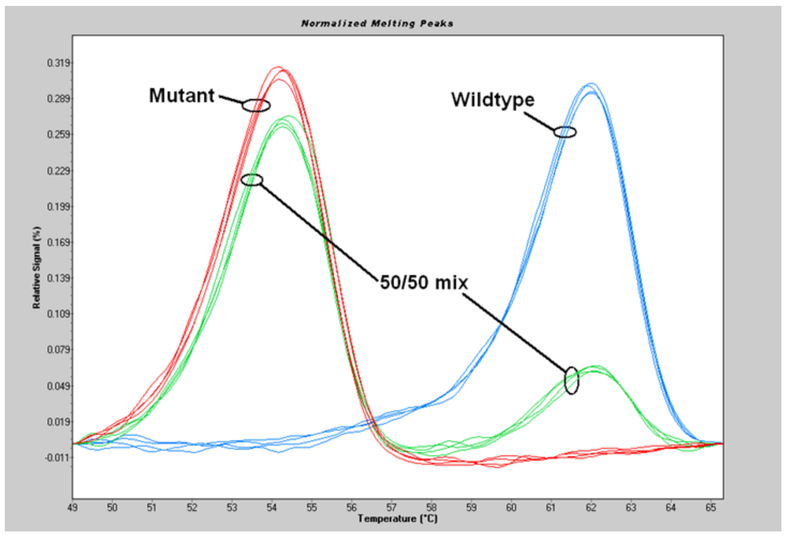


Figure 8

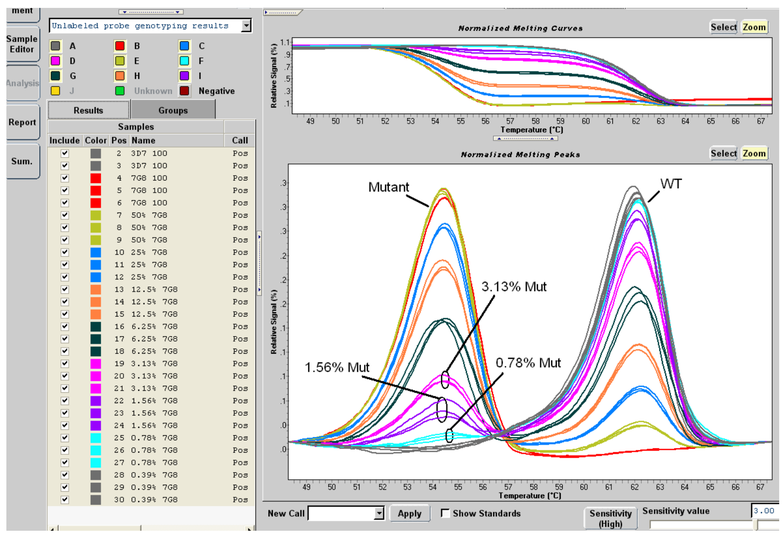
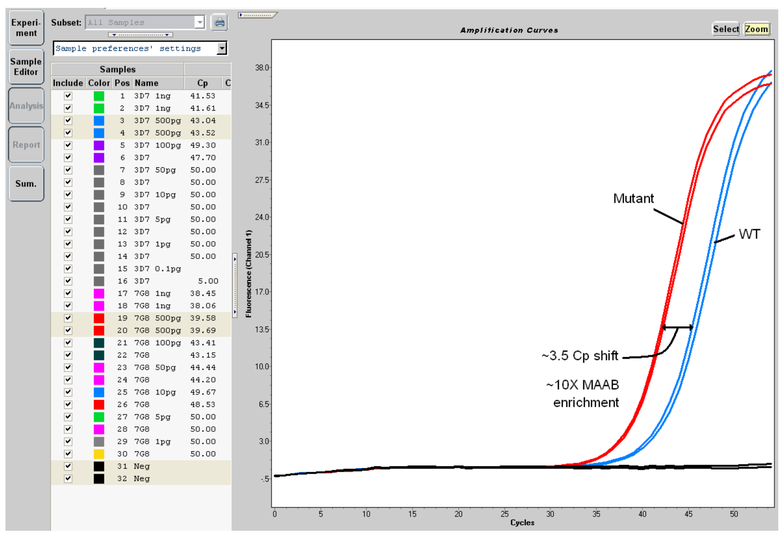


Figure 9



CONCLUSIONS

The following criteria have a significant impact on the ability to perform MAAB with LunaProbes: 1. Temperature transition rate between PCR target temperatures; 2. Annealing temperature relative to the probe Tms; and 3. Exonuclease negative polymerase. Applying these criteria to the targets in this study, a detection sensitivity of approximately 1% of the mutant allele was achieved. Based on the real time PCR crossing points, a MAAB enrichment factor of 10X could be estimated. Applications of this method include detection of known somatic mutations (p53, EGFR, BRAF), early identification of parasitic infections contraindicating standard therapy, and high sensitivity detection of fetal DNA mutations. Concomitant analysis of real time PCR and high resolution melting data, on the LS32 instrument, coupled with the rapid cycle PCR approach appear to be critical to performing MAAB using LunaProbe assays.