Application Forum

Mutant Allele Amplification Bias 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 heterogzygous sequence variants. Based on the use of dsDNA saturating dyes, Hi-Res Melting is capable of detecting SNP's and In/Del's in amplicons up to 400 bp at a sensitivity >99%. Since its introduction in 2003, additional applications for Hi-Res Melting have been developed, including genotyping for known sequence variants using small amplicons or unlabeled probes (LunaProbes). LunaProbe's are blocked on the 3′ end to prevent extension during PCR and use the dsDNA saturation dye LCGreen Plus to discriminate the genotype of the allele 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.

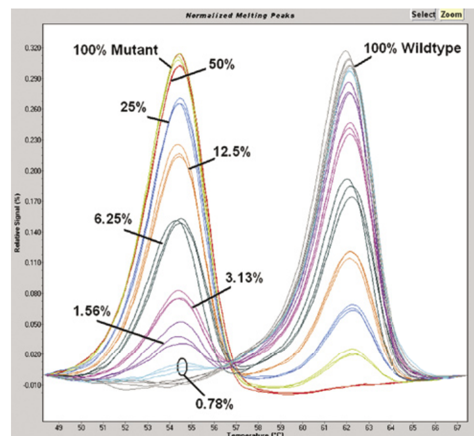

Figure 1. Normalized derivative peaks from LunaProbe. Wild-type allele (blue) = 62°C. Mutant allele (red) = 54°C. An annealing temperature of 58°C was used to bias the amplification of the mutant allele in the 50-50 mixed samples (green). This amplification bias allows greater resolution of the mutant allele by a factor of ~10× and sensitivity down to 0.7–1.5%.

METHODS

We investigated the use of LunaProbes to discriminate the mutant allele at ≤5% in a background of the wild-type allele. Because LunaProbes are inherently large (25–30 bp), in order to generate sufficient fluorescent signal, they present a unique opportunity to preferentially bias amplification of the mismatch allele. Mutant allele amplification bias (MAAB) is achieved by setting the annealing temperature of PCR such that it is approximately half way between the Tm of the perfectly matched and mismatched probe. At this mid-Tm annealing temperature, the perfect match probe (wild-type allele) is bound to its target and is stable enough to partially retard amplification. Rapid cycle PCR performed on the LightScanner 32 (LS32) was required to maintain the stringency of the target annealing temperature and hinder amplification of the wild-type allele. In contrast, the slower temperature transition rates of a conventional thermal block cycling instrument were not practical for MAAB.

RESULTS

Detection sensitivity of the mutant allele without MAAB was determined to be 5%. Based on the observed Tm's of the LunaProbe (62° and 54°C for the wild-type and mutant alleles, respectively), an annealing temperature of 58°C was used to induce MAAB in a dilution series of mixed samples. MAAB was not observed when the same approach to setting the annealing temperature of PCR was performed on a standard thermal block cycler, presumably due to the slower transition rates between annealing and denaturation temperatures.

CONCLUSIONS

Rapid cycle PCR was critical for inducing MAAB over the wild-type allele in samples that were mixed at 50% of each allele. A MAAB factor of approximately 10× allowed discrimination of the mutant allele down to 0.7–1.5%. Applications of this method include detection/confirmation of common somatic mutations (p53, EGFR, BRAF) and early identification of mutant bacterial infections (malaria) where standard therapies are contraindicated. The ability to use a combination of real time PCR data and Hi-Res Melting on the LS32 instrument, coupled with the rapid cycle PCR approach appear to be critical to performing MAAB using LunaProbes.

For further information visit our web site at www.idahotech.com or contact us at 1-800-735-6544: Jason T. McKinney, Life Science Projects Manager, Idaho Technology Inc.