Application Forum

Small Amplicon Genotyping Using Internal Temperature Calibration and High Resolution-Melting

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INTRODUCTION

Small amplicon genotyping is a simple alternative to probe-based methods and more rapid and cost-effective than sequencing. This approach relies on the thermodynamic differences between two molecules that differ by potentially only a single base. Because there are no custom probes (labeled or unlabeled) required, assay design and optimization are simplified, making this technique rapid and inexpensive. The technique relies on the placement of standard, unmodified PCR primers immediately adjacent to, or very near, the SNP or sequence variant of interest, thus generating an amplicon of 40-60 base pairs. A double-stranded DNA specific binding dye with saturation characteristics (i.e., LCGreen Plus® dye, Idaho Technology, Inc., Salt Lake City, Utah, USA) is included in the PCR reaction and is the source of fluorescent signal in the reaction. The method of high-resolution melting is employed post-PCR to thermally denature the small amplicon and measure the subtle differences in melting temperature (Tm) between both homozygous and heterozygous genotypes. Depending on the type of SNP, the difference in Tm between the two homozygous alleles can vary from 0.1°C up to 1.5°C. Theoretically, amplicons generated from the so-called “base-pair neutral” homozygotes (CC>GG or TT>AA) vary little, if any in Tm, making accurate genotyping problematic, regardless of the platform. With the recent development of a High Sensitivity Genotyping Mastermix, which includes both the saturating dye LCGreen and two internal temperature calibration dsDNA fragments (two sets of complementary oligonucleotide sequences with low and high Tms), even these base-pair neutral changes can be reliably differentiated based on Tm, allowing for sensitive and accurate genotyping in a high-throughput and cost-effective fashion.

Regular unmodified primers obtained at any standard synthesis facility produce excellent results. HSGM contains calibrators melting at approximately 62°C and 92°C. It is important to design the amplicon so that neither calibrator interferes with product melting. In general, as long as the amplicon is 45-60 base pairs and between 25-60% GC, the majority of designs are compatible. Adjuvants such as betaine or DMSO can be used, but will affect (i.e., decrease) the Tm of the calibrator molecules as well as the amplicon, so their use should be dictated by the need to achieve robust, specific PCR product. PCR optimization is streamlined by running primers at equimolar concentrations (as opposed to asymmetric in some probe-based methods) across an annealing temperature gradient, and using both high-resolution melt and agarose gel results to identify the optimal primer annealing condition. The annealing condition with the cleanest PCR product by gel or melting curves, and highest melting fluorescence signal change is typically the best choice. After a candidate PCR condition is identified, it is essential to run multiple samples in duplicate using the chosen protocol, ideally using samples with known genotypes as controls for the experiment.

Figure 1. Raw melt data showing position of calibrators relative to amplicon.
Figure 1 shows a 52 base-pair fragment amplifying a base-pair neutral SNP (A>T). Amplification conditions were one cycle of 95°C/2 min followed by 45 cycles of two-step PCR using 94°C/30 sec, 66 anneal/extension/30 sec. A final denature and anneal of the samples was performed at 94°C/30 sec and 28°C/30 sec. Primers were added at 0.1 μM final concentration. Following amplification, the BioRad 96-well plate was transferred by hand into the LightScanner for melting analysis. A melting protocol was run starting at 50°C and ending at 96°C. Genotyping accuracy without calibration was 75%, whereas after calibration 64/64 correct results were obtained. Samples were run in duplicate, represented once on each half of the plate (i.e., Sample A1 = Sample A7, etc.) as seen by the pattern of calibrated genotype calls. Samples 5D and 11D were no-template controls.

Only with internal oligonucleotide-based calibration can certain melting limitations be overcome. For example, sample buffer differences modify the local environment and influence kinetics and equilibria—resulting in altered intersample melting temperatures and inaccurate genotype calls. In addition, volume differences introduced by the user can make higher volume reactions appear to be higher Tm due to increased thermal mass and decreased rate of heat transfer to the sample. This is unavoidable unless a thermocouple is placed within each reaction, which is not practical. Both of these perturbances can be mitigated with this internal calibration system, enabling more accurate genotyping. In our hands across many different SNPs (including base-pair neutral SNPs) greater than 98% sensitivity and specificity were obtained with minimal manual review of data. Using the high-sensitivity setting, automated software calls were correct >95% if the time. Using the guide-lines and protocol described in the Idaho Technology Small Amplicon Genotyping Technical Note (www.idahotech.com/LightScanner), several users have experienced similar genotyping success rates.

Internal calibrators are an attractive and effective way to improve genotyping using high-resolution melting of small amplicons (40-60 bp). Like other high-resolution melting applications, small amplicon genotyping is homogenous and simple. Internal calibrators can effectively counteract inter-sample variability due to both physical and chemical factors. Small amplicon genotyping with internal calibration allows accurate genotyping of the most thermal neutral base changes (i.e., G>C or A>T), and improves the melting temperature discrimination of all SNPs.

For more information on genotyping solutions offered by Idaho Technology, see our website http://www.idahotech.com/Genotyping/.