INTRODUCTION

Small Amplicon Genotyping (SAG) and LunaProbes are well-established Hi-Res Melting genotyping methods. SAG assays require two primers that are used in equal concentrations creating symmetric amplification of the target sequences. The melting peak is created by thermal denaturation of the small amplicon (40-100 bp) in the presence of LC Green Plus dye.

LunaProbe assays require a 3' blocked oligonucleotide, which serves as an unlabeled probe, in addition to primers that create an amplicon ranging from 40-200 bp. In order to generate sufficient probe signal, the primer must be used in unequal concentrations to create asymmetric amplification of the probe target strand. This allows the LunaProbe to hybridize to the single stranded copies producing a probe melting peak which is used to distinguish between alleles.

A new method, Snapback primers, shows promise as an alternative genotyping technique. Snapback assays utilize two primers, similar to LunaProbe assays, however the second primer is modified by attaching the “probe” element to the 5’ end of the primer. This probe element is complementary to the targeted SNP region and distinguishes between alleles in much the same manner as a LunaProbe. LunaProbes and Snapback assays produce a probe melt (relatively low Tm) and an amplicon melt (relatively high Tm) region, while SAG produces a single amplicon melt only. (See Figure 1)

MATERIALS AND METHODS

Ten SNPs were interrogated using each of the three genotyping methods. Comparisons included genotype accuracy, discrimination of SNP allele frequency, melting profile and amplicon size. In the SAG assays, two primers are used in equal concentrations creating symmetric amplification of the small amplicon (40-100 bp) size. The LunaProbe assay used appropriate asymmetric primer concentrations and were included in the PCR reaction. The LunaProbe primers were used in excess via asymmetric primer concentrations and creates a probe-DNA duplex which upon thermal denaturation generates the probe melting profile.

The Snapback assays utilized the same primers as the LunaProbe assays, but one of the primers was modified with a 5’ probe element. This 5’ probe element performs intermolecular hybridization with its own DNA single strand and produces a melt profile similar to a LunaProbe. The primer concentrations can be modified to create asymmetric amplification in the forward or reverse direction depending on the target sequence and the SNP. The probe element can be linked to either primer so it can hybridize to the forward or the reverse strand allowing for design flexibility and faster assay optimization.

RESULTS AND DISCUSSION

The ability of a genotyping method to genotype depends on creating an small amplicon as possible. The SAG Between the primers has a significant effect on the thermal stability and melting behavior of the amplicon. (See Figure 2). The small amplicons genotypes use high sensitivity labelled hts containing internal temperature calibrators. The calibrators Tm peaks bracket the amplicon melt, enabling the software to baseline all of the samples in that experiment to the calibrators. The results Tm differences caused by the SNP can now be more separated and genotyped with a high degree of confidence and repeatability.

When designing LunaProbes and Snapback assays it is important to note that the forward or reverse DNA strand can be amplified by varying the primer concentrations. In some cases you can get a better separation of 3Tm of unequal allele strands and specificity of design generating any time ranging from 87 to 146 bp. (See Table 1) The LunaProbe amplicon size ranged from 75-124 bp. The LunaProbe itself hybridizes to its target strand (generated in excess via asymmetric primer concentrations) and creates a probe-DNA duplex which upon thermal denaturation generates the probe melting profile.

The Snapback assays utilized the same primers as the LunaProbe assays, but one of the primers was modified with a 5’ probe element. This 5’ probe element performs intermolecular hybridization with its own DNA single strand and produces a melt profile similar to a LunaProbe. The primer concentrations can be modified to create asymmetric amplification in the forward or reverse direction depending on the target sequence and the SNP. The probe element can be linked to either primer so it can hybridize to the forward or the reverse strand allowing for design flexibility and faster assay optimization.

CONCLUSIONS

In this study we first designed the primers for the LunaProbe assays. Then we optimized the same primer sets for the Snapback assays. Nine of the ten primer sets were successfully converted to Snapback assays. The results show that all three genotyping chemistries can genotype correctly with LunaProbes having the best allele separation.

Comparison of LightScanner 32 vs. LightScanner (See Figure 3)

The all GenoScan assays were converted to run on the LightScanner instrument (LS32, Idaho Technology, Inc.) The LS32 is a 20-sample instrument capable of real-time PCR and high resolution melting. The LS32 is compatible with many industry-standard chemistries including TagView, HiMelting, and Snapback assays.

Allele Fraction

We investigated allele fraction detection using both the LightScanner and the LightScanner 32. The ability to detect small fractions (i.e. 0.5%) of the mutant allele is important for applications such as somatic mutations and in pathogen detection. The LunaProbe and SAG assays performed with the best sensitivity, showing good detection with artificially spiked allele fractions of 5% on both the LightScanner and the LightScanner 32. (See Figure 4)