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Systematic comparison of High-Res Melting® genotyping methods on the LightScanner® and LightScanner 32: Small Amplicons, Lunaprobes™, and Snapback Primers

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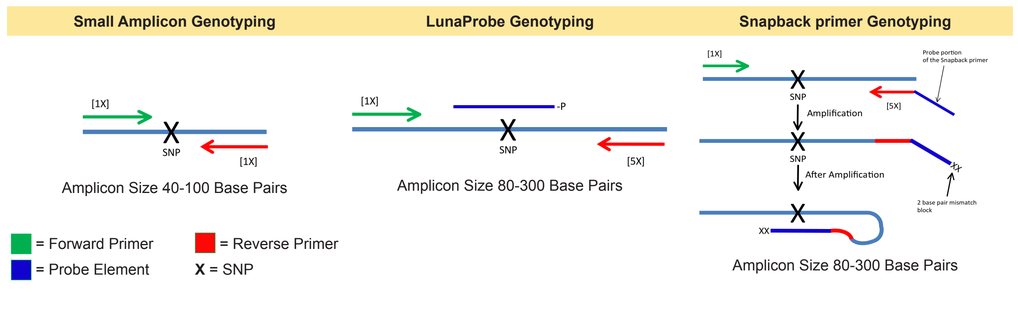
INTRODUCTION

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 sequence. The melting peak is created by thermal denaturation of the small amplicon (40-100 bp) in the presence of LCGreen 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 80-200 bp. In order to generate sufficient probe signal, the primers 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 also use 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 complimentary to the targeted SNP region and distinguishes between alleles in much the same manner as a LunaProbe. LunaProbe 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)

Figure 1: Assay design strategies and considerations for (A) SAG (B) LunaProbe and (C) Snapback genotyping methods.



MATERIALS AND METHODS

Ten SNPs were interrogated using each of the three genotyping methods. Comparisons included genotype accuracy, discrimination of unequal allele fractions and simplicity of design generating amplicons ranging from 87 to 149 bp. (See Table 1)

SAG assays used primers placed as close as possible to each SNP, generating amplicons in the 41-66 bp range. The SAG assays benefited from the use of Idaho Technology High Sensitivity Master Mix with internal temperature calibrators.

The LunaProbe assays used appropriate asymmetric primer concentrations and were included in the PCR reaction. 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 intramolecular hybridization with it's 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.

Table 1

Method	rs#	Amplicon Size	SNP	Ancesetral Allele	Primer Ratio (Fwd:Rev: Probe)	Sensitivity Required for 100% Correct Genotyping	Average Sensitivity Required
Small Amplicon Genotyping	rs1573496	46	C/G	C	1:1	Normal, -2	Normal, = 3.1
	rs1726866	42	C/T	C	1:1	Normal, -3	
	rs2236142	55	C/G	C	1:1	Normal, -3	
	rs2241894	41	A/G	G	1:1	Normal, - 5	
	rs3762894	51	C/T	T	1:1	Normal, - 3	
	rs6005863	46	A/G	G	1:1	Normal, -3	
	rs9608698	45	C/G	G	1:1	Normal, -3	
	rs1031825	66	A/C	C	1:1	Normal, -2	
	rs7566605	51	C/G	G	1:1	Normal, -4	
	rs1382387	50	G/T	G	1:1	Normal, -3	
LunaProbe	rs1573496	79	C/G	C	5:1:5	Normal, -4	Normal, = 3.6
	rs1726866	91	C/T	C	5:1:5	Normal, -4	
	rs2236142	117	C/G	C	5:1:5	Normal, -4	
	rs2241894	82	A/G	G	5:1:5	Normal, -4	
	rs3762894	108	C/T	T	1:5:5	Normal, -4	
	rs6005863	89	A/G	G	5:1:5	Normal, -4	
	rs9608698	86	C/G	G	5:1:5	Normal, -1	
	rs1031825	124	A/C	C	1:5:5	Normal, -4	
	rs7566605	75	C/G	G	1:5:5	Normal, -3	
	rs1869458	98	C/G	C	5:1:5	Normal, -4	
Snapback	rs1573496	96	C/G	C	5:1	Normal, -4	Normal, = 2.9
	rs1726866	116	C/T	C	5:1	Normal, -3	
	rs2236142	143	C/G	C	5:1	Normal, -1	
	rs2241894	106	A/G	G	5:1	Normal, -3	
	rs3762894	124	C/T	T	1:5	Normal, -4	
	rs6005863	102	A/G	G	5:1	Normal, -4	
	rs9608698	113	C/G	G	5:1	Normal, -1	
	rs1031825	145	A/C	C	1:5	Normal, -4	
	rs7566605	87	C/G	G	5:1	Normal, -2	
	rs1382387	149	G/T	G	1:5	Normal, -3	

Figure 2: Normalized derivative melting peaks of all assays using (A) SAG (B) LunaProbe and (C) Snapback. All asssays were amplified using a BIO-RAD My IQ instrument. The Hi-Res Melting and data analysis were performed on the LightScanner and LS32 instruments and software.

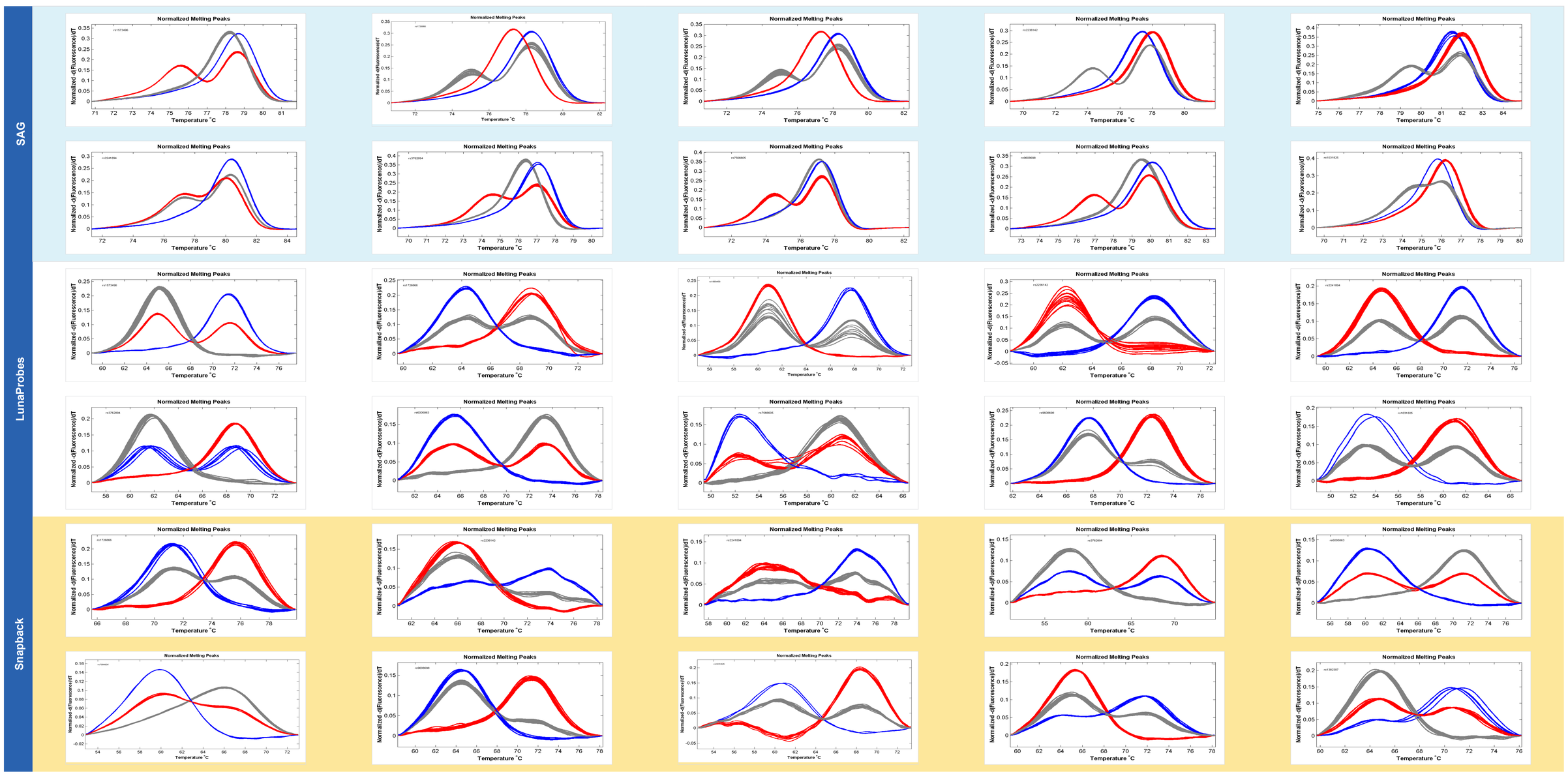


Figure 3: LightScanner and LS32 assay comparison (RS1573496).

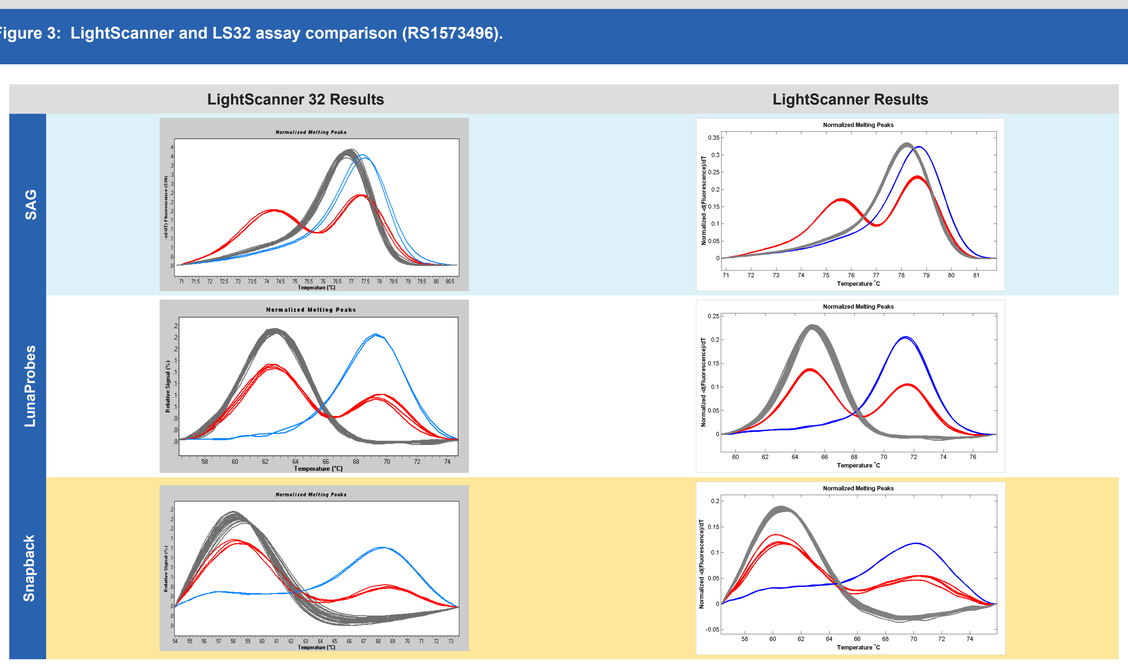
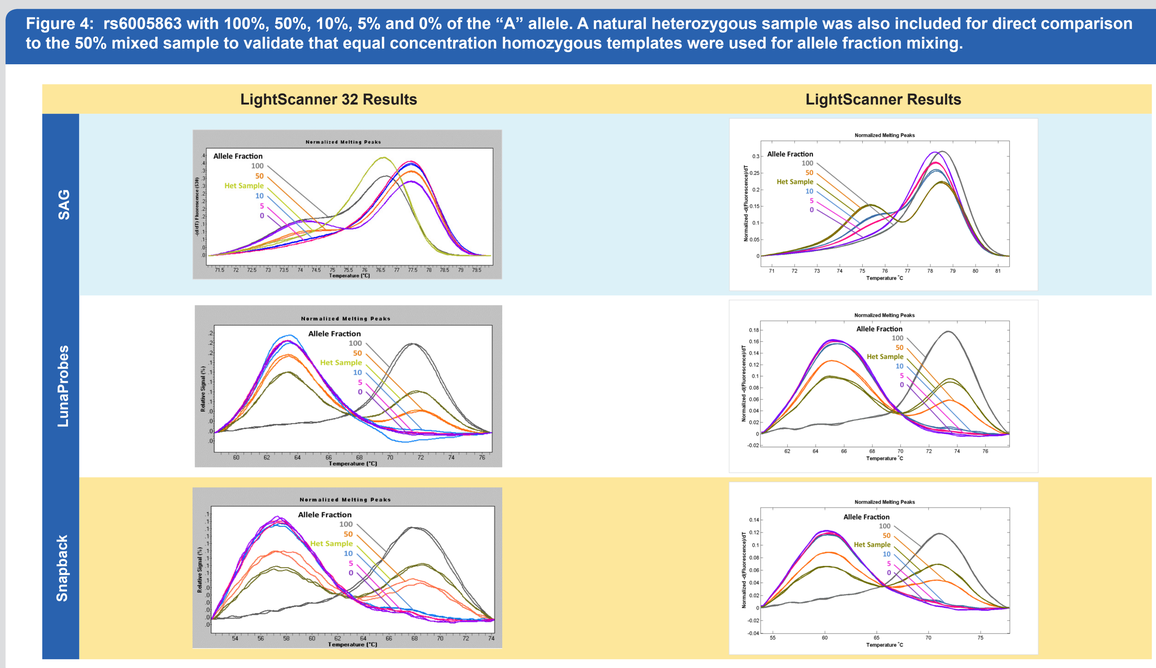


Figure 4: rs6005863 with 100%, 50%, 10%, 5% and 0% of the "A" allele. A natural heterozygous sample was also included for direct comparison to the 50% mixed sample to validate that equal concentration homozygous templates were used for allele fraction mixing.



RESULTS AND DISCUSSION

The ability of SAG to correctly genotype depends on creating as small an amplicon as possible. The SNP between the primers has a significant effect on the thermal stability and melting behavior of the amplicon. (See Figure 2) The small amplicon genotyping assays use High Sensitivity Master Mix containing internal temperature calibrators. The calibrator Tm peaks bracket the amplicon melt, enabling the software to adjust all of the samples in that experiment to the calibrator peaks. The minute Tm differences caused by the SNP can now be separated and genotyped with a high degree of confidence and repeatability.

When designing LunaProbe and Snapback assays it is important to note that the forward or the reverse DNA strand can be amplified by varying the primer concentrations. In some cases you can get a better separation of ΔTm the LunaProbe or Snapback peaks from the forward DNA strand vs. the reverse DNA strand due to the SNP base change, secondary structure and nearest neighbor effects.

Snapback assays require more attention to detail during the design process. The Snapback probe element needs to be the compliment sequence of the target SNP region. Additionally, to prevent extension of the snapback primer it is important to include at least 2 mismatched bases to its 5' end. When properly designed and optimized, snapback assays give distinct and well defined melting peaks for the different genotypes.

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

The rs1573496 assay was converted to run on the LightScanner32 instrument. (LS32, Idaho Technology, Inc.) The LS32 is a 32-sample instrument capable of real-time PCR and high resolution melting. The LS32 is compatible with many industry standard chemistries including TaqMan®, HybProbes, and SimpleProbes.

Allele Fraction

We investigated allele fraction detection using both the LightScanner and the LightScanner 32. The ability to detect small fractions (i.e. 2-5%) 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 LS32. (See Figure 4)

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 and ease of calls (Table 1, Avg Sensitivity Required) followed by SAG and Snapback. A key factor in the success of any assay using PCR is primer design. One way to asses the quality of the primer set is to amplify them using an annealing temperature gradient. In most cases a primer set that produces a single amplicon peak over a wide range of annealing temperatures is amenable for use with other chemistries.