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Detection of Mutant Allele Fractions as low as 0.01% by combining Digital PCR, LunaProbes™ and Mutant Allele Amplification Bias (MAAB) Methods.

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INTRODUCTION

Detection of low level mutants has driven the development of several intriguing methods. We have combined aspects of three existing techniques: digital PCR, LunaProbes, and Mutant Allele Amplification Bias (MAAB) (**see Figure 1**). Digital PCR (Vogelstein and Kinzler, 1999) requires many replicates of a sample using template diluted to 1 copy/reaction. A variation on the original digital PCR technique was developed for Hi-Res Melting® (HRM) using template dilutions of 5 template copies/ reaction (McKinney, 2007). This dictates the mutant allele, if present, will comprise at least 20% of alleles in a reaction and enable detection of heteroduplexes. Typically the sensitivity of digital PCR is approximately 2%. The sensitivity can be increased, depending on the number of replicates assayed. LunaProbe assays add an unlabeled oligonucleotide that is 3' blocked in the PCR reaction (Zhou, 2005). LunaProbes add genotyping capabilities in addition to amplicon scanning when using a HRM instrument (see figure 2). The sensitivity of LunaProbes has been established at approximately 5% (Wall, 2007). MAAB utilizes the increased stability of the LunaProbe when it is hybridized to the perfectly matched wildtype (WT) allele verses the decreased stability of the LunaProbe hybridizes to the mismatched mutant allele to preferentially amplify the mutant allele. MAAB has demonstrated sensitivity of <1% (McKinney, 2009). In this experiment we combined these methods to increase the sensitivity and usefulness of HRM.

Figure 1: Digital PCR, LunaProbes, and Mutant Allele Amplification Bias (MAAB)

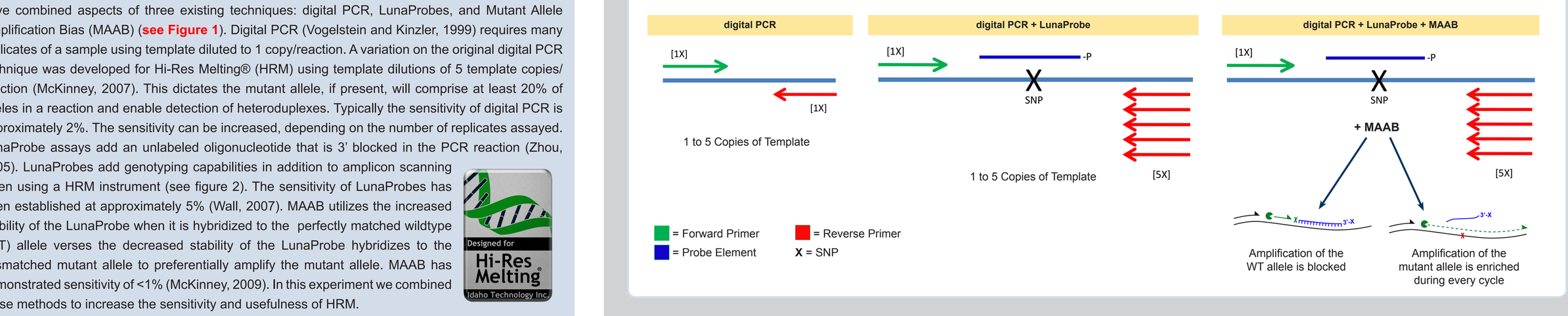
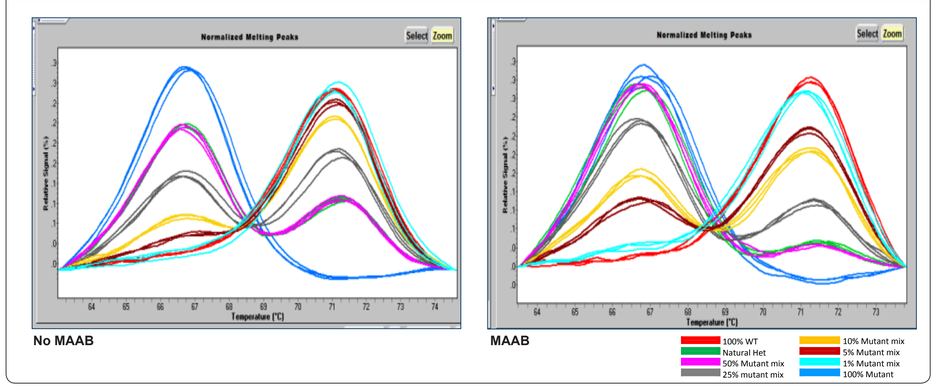


Figure 2: Mutant allele fraction titration comparing a normal PCR protocol vs. a MAAB PCR protocol.



MATERIAL AND METHODS

A SNP in the phenylalanine hydroxylase exon 11 (PAHx11) gene was targeted with a standard LunaProbe assay. Homozygous samples for both alleles were identified. The homozygous WT and homozygous mutant samples were standardized for concentration (15ng) and then mixed to generate serial dilutions of the mutant allele down to 0.01%. The 1%, 0.1% and the 0.01% allele fraction samples were then serially diluted to 5 - 100 copies. The experiments were run using a 96 well plate of each of 1%, 0.1%, and 0.01% mutant allele fraction at each concentration using digital PCR, digital PCR + LunaProbes and digital PCR + LunaProbes + MAAB protocols. Every experiment included two replicates of 100% WT and 100% mutant allele samples at 15ng/reaction and two no template controls. The MAAB and digital PCR + LunaProbes + MAAB experiments used an Eppendorf Realplex^4 Master Cycler Pro S (Eppendorf, Westbury, New York) with an annealing temperature of 57°C to achieve MAAB conditions. The Eppendorf instrument has a temperature transition rate of up to 6°C/s. The faster temperature ramp rates are crucial when performing PCR experiments with MAAB. At the annealing step of PCR the faster ramp rate allows the probe to hybridize to their target region and effectively block the extension of the primer of the WT allele. The faster ramp rate also helps to reduce amplification of the WT allele during the denature step by reducing the time the polymerase has to amplify the WT allele after the probe has melted off the WT allele strand. The digital PCR and digital PCR + LunaProbe assays were run on a Bio-Rad IQ thermal cycler (Bio-Rad, Hercules, CA). With an annealing temperature of 68°C and an extension step at 72°C to eliminate any allele bias. After amplification the samples were melted on the 96 well LightScanner® (Idaho Technology Inc., Salt Lake City) (**see Figure 2**).

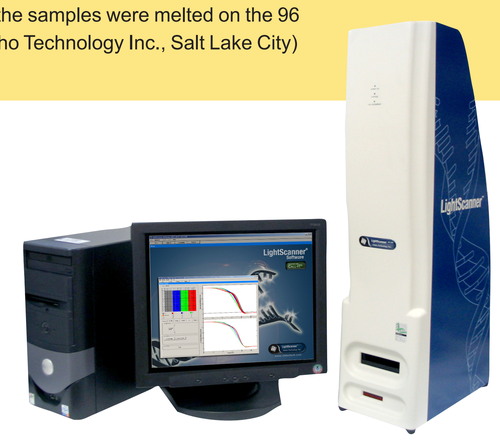
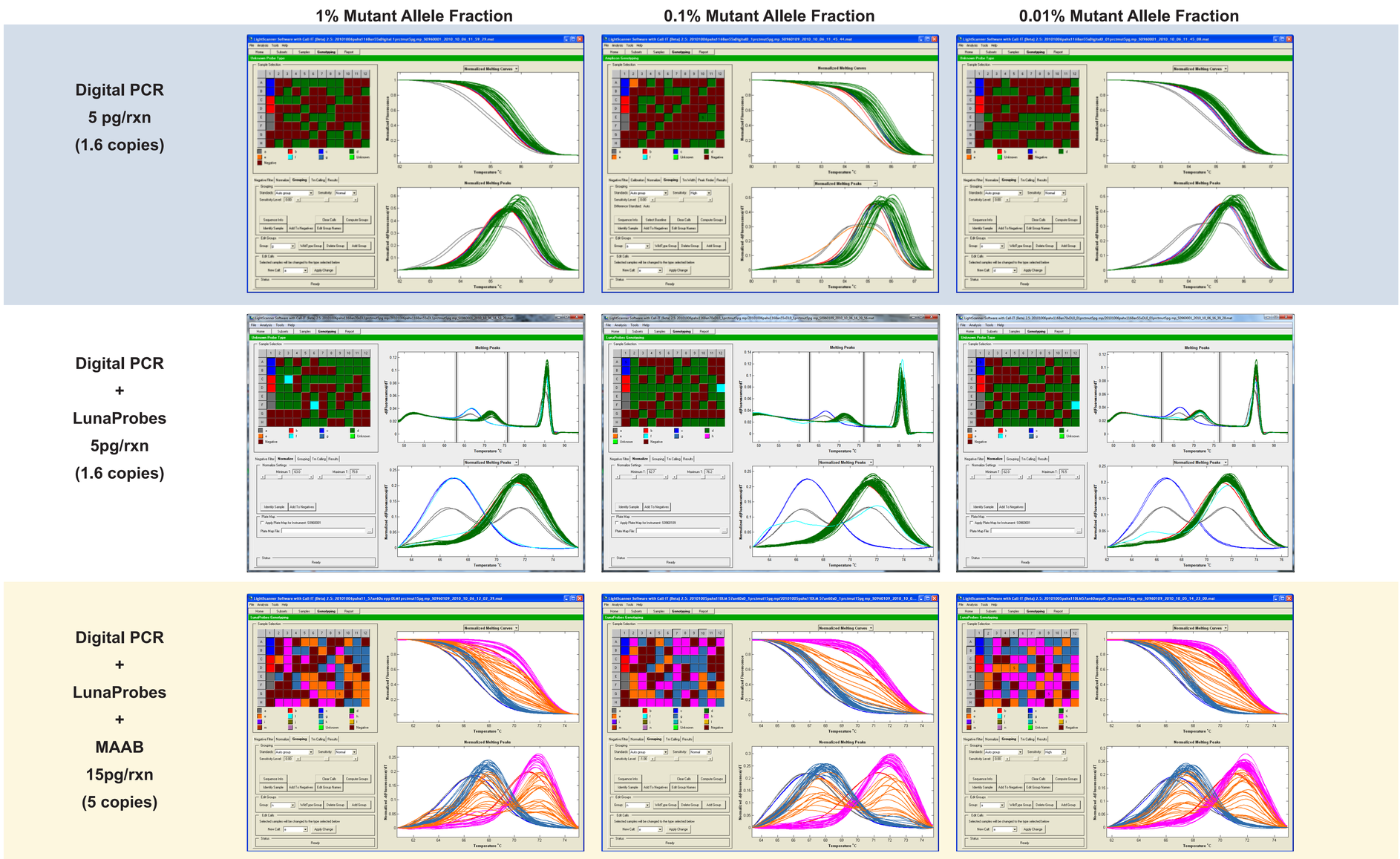


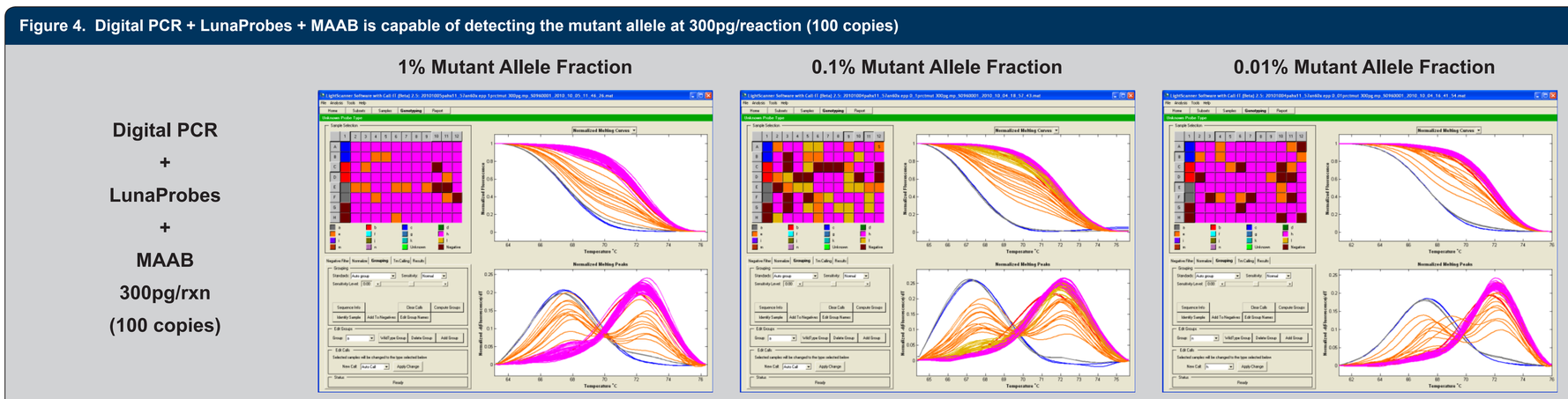
Figure 3. A comparison of digital PCR, digital PCR + LunaProbes and digital PCR + LunaProbes + MAAB at detecting low mutant allele fractions



RESULTS AND DISCUSSION

The digital PCR method used 5pg/reaction (approximately 1.5 copies) of template. Digital PCR was limited by only having the amplicon peak to analyze. Digital PCR did amplify the target successfully at this dilution and was able to detect 1 heterozygous sample in the 0.1% mutant allele fraction. In the other allele fraction experiments digital PCR was unable to detect any mutant allele samples. The addition of LunaProbes improved digital PCR's sensitivity and was able to detect a mutant allele fraction in each of the mutant allele fraction mixes. The digital PCR with LunaProbes and MAAB showed increased ability to detect the mutant allele with 42 samples detected in the 1% mutant mix, 37 samples detected in the 0.1% mutant mix and 43 samples detected in the 0.01% mutant mix (**see Figure 3**). Although digital PCR with LunaProbes and MAAB is technically not digital the template copy number is considerably less than the amount of template used in normal PCR. MAAB has the ability to increase the detection limits of mutant alleles in a high WT allele background. The digital PCR with LunaProbes and MAAB technique can be used on template concentrations of up to 300pg (100 copies/reaction) and still detect mutant allele fractions of 0.01% effectively (**see Figure 4**).

Figure 4. Digital PCR + LunaProbes + MAAB is capable of detecting the mutant allele at 300pg/reaction (100 copies)



CONCLUSIONS

The ability to detect low levels of mutant DNA has multiple applications. Some of these include detection of drug resistant pathogens, noninvasive prenatal screening, low level somatic mutations and detection of residual disease from peripheral blood post-therapy. Combining digital PCR, LunaProbes and MAAB increases sensitivity 10-100 fold and allows mutant allele confirmation via specific probe melting profiles reducing the need and expense of sequencing.