

Detection of BRAF v600e to 0.1% Using Unlabeled Probes and High Resolution Melting through Amplification Bias.

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INTRODUCTION

High Resolution Melting (HRM) is a set of post-PCR genetic screening techniques to rapidly detect mutations while reducing sample manipulation. HRM techniques are non destructive, allowing confirmatory sequencing when desired. HRM is highly suited for known mutation screening or targeted genotyping. The v-raf murine sarcoma viral oncogene homolog B1 (BRAF) v600e is a proto-oncogene mutation linked to many cancers including colorectal, thyroid, and melanomas. Diagnosis is made by taking biopsies of suspected tissue. As biopsies obtain varying amounts of cancerous tissue, a highly sensitive and accurate assay for the v600e mutation is required. This study highlights the ability of allele biased HRM techniques to identify the BRAF v600e mutation in varying wild-type allele backgrounds.



METHODS

This study was performed using the LightScanner@96 and the LightScanner@32 instruments (Idaho Technology, Inc.). Unlabeled probe (LunaProbes™) assays use three oligonucleotides, two target-specific primers and an unlabeled probe, and asymmetric PCR in which the forward and reverse primers are present in unequal concentrations (Figure 1). The probe is blocked on the 3' end with an amino modifier (IDT). It is important to note that the probe must be perfectly matched to the wild type allele. This assay used LightScanner® Master Mix that includes the double stranded DNA dye LCGreen@Plus+ (Idaho Technology, Inc.). Each reaction had a final volume of 10µL with a 2 µL of mineral oil overlay added to the reactions on the LightScanner32 and a 20 µL mineral oil overlay on the LightScanner 96 instruments.

The LunaProbes HRM technique uses a double stranded DNA dye called LCGreen Plus+ which fluoresces when the DNA is hybridized and loses fluorescence when the DNA disassociates and becomes single stranded. Genotypes are identified by detecting changes in hybridization energies of the probe melting profile, which can be displayed as melting peaks, curves or difference plots. High resolution capable instruments need a high degree of temperature homogeneity and a high rate of acquisitions per second.

The introduction of the 3' blocked probe element can in some cases (assay specific) be used to differentially amplify the mutant allele using a mutant allele amplification bias (MAAB) PCR technique to increase the mutant allele signal (Figure 2). LunaProbe assays can be carefully optimized to favor the amplification of the mutant allele (Figure 3). Mutant allele amplification bias assays need to have very robust primers that amplify the desired product over a wide range of annealing temperatures because the mutant allele amplification biased protocol deliberately uses suboptimal primer annealing temperatures in order to force the amplification of the mutant allele. A fast way to test primers is to run an annealing gradient starting 10-15°C below the predicted T_m of the primers. The probe should have a predicted T_m above the primers so that it will hybridize to its target before the primers. The probe is designed to perfectly match the wildtype allele. In this study, allele blocking techniques were employed to identify the BRAF v600e mutant genotype in a wildtype background. This mutant allele amplification bias was accomplished by designing the probe to perfectly match the wild-type allele and carefully adjusting amplification conditions to preferentially amplify samples containing the BRAF v600e mutation. Varying allele fractions were tested in a wild-type genetic background to determine the lowest detected allele fraction.

Figure 1: LunaProbes Assay Design and MAAB Technique

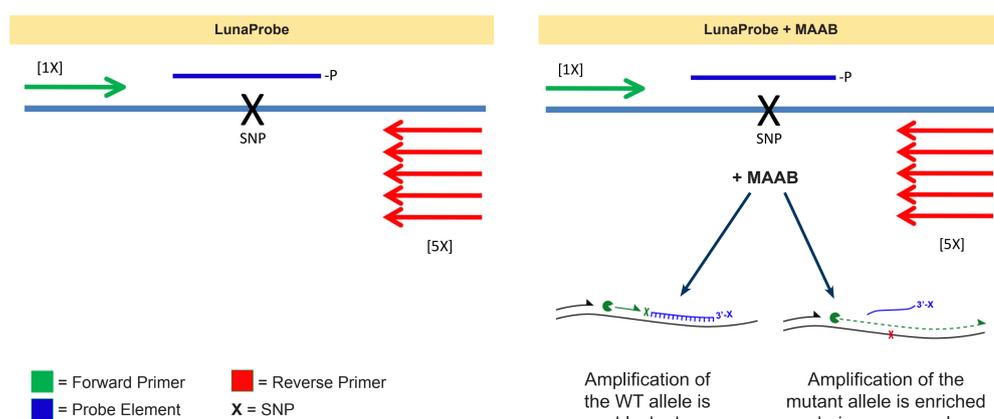


Figure 2: LunaProbes advantages and considerations.

- Advantages:

- Amplicon size ranges from 75-300bp
- Gives a probe peak (for genotyping) and an amplicon peak (for scanning)
- Can detect mutant allele fractions down to 5%
- Using MAAB can detect mutant allele down to 0.1%

- Considerations:

- Requires Asymmetric PCR
- Requires 3 oligos (2 primers and a probe)
- The probe must be blocked (costs more to synthesize)
- Requires fast temperature ramp rates and precise temperature control

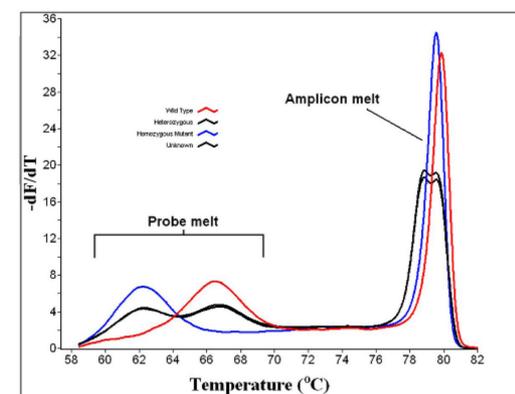
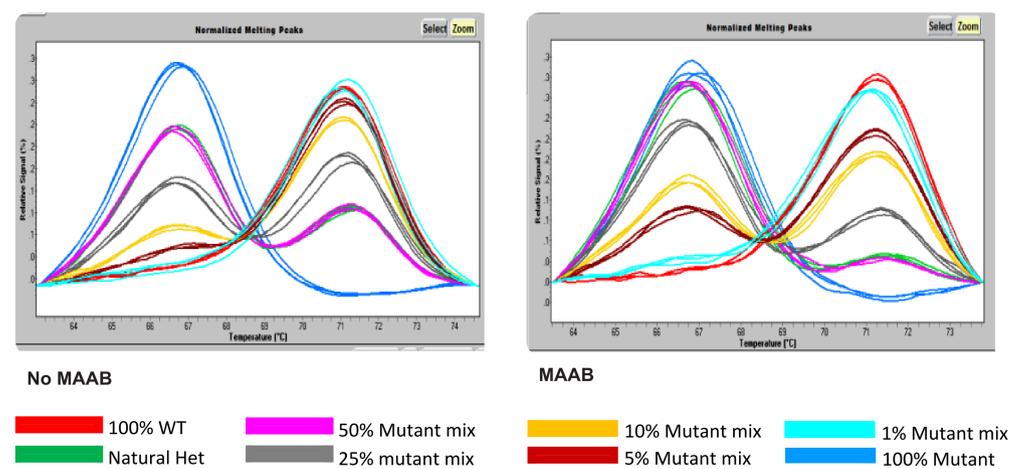


Figure 3: Mutant allele fraction titration comparing a normal PCR protocol vs. a MAAB PCR protocol.



This figure shows that the mutant LunaProbe Peak signal is increased when mutant allele amplification techniques are used.

RESULTS

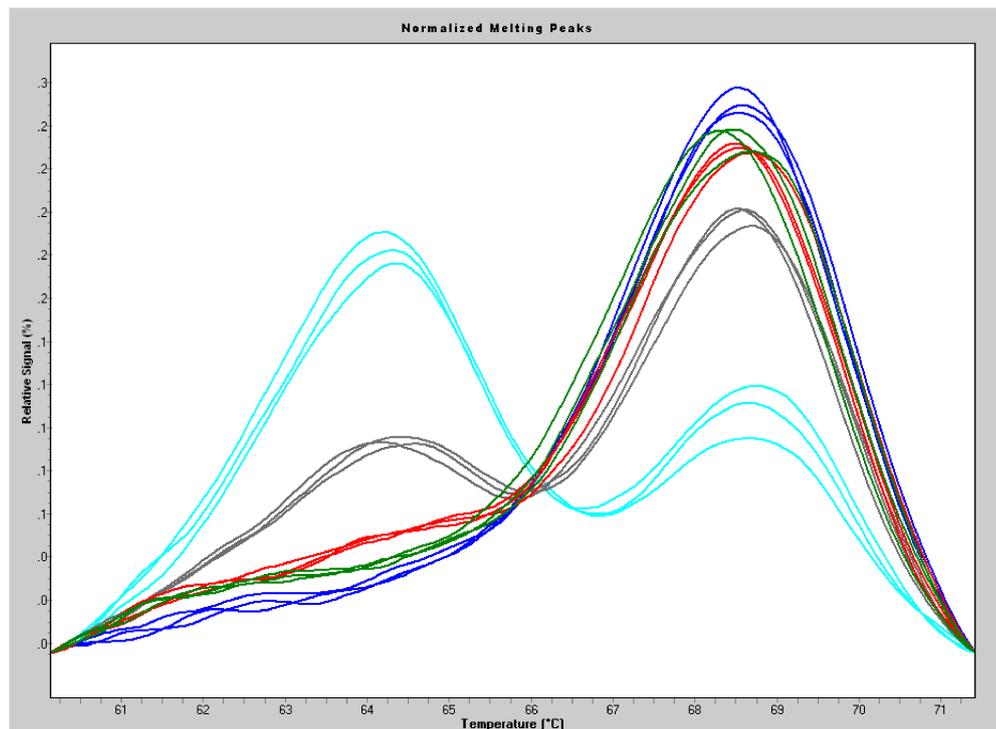
This assay combined LunaProbe and mutant allele amplification enhancing techniques to achieve extremely high sensitivity detecting the BRAF v600e mutation in a homogenous (closed system) assay requiring no post-PCR manipulations. This greatly reduces the risk of post PCR contamination and sample manipulation errors. The mutant allele amplification bias resulted in detection of BRAF v600e mutation at 0.1% allele fraction (Figure 4).

CONCLUSIONS

This study shows that HRM, in combination with robust assay optimization and precise temperature control, can identify the BRAF v600e mutation as low as 0.1% mutant allele fraction. As HRM instruments improve, mutation detection at low levels with higher throughput will be achievable. This technique can be applied to many genotyping applications such as drug resistance, drug efficacy testing, cell free fetal DNA screening, detection of low level somatic mutations of residual disease from peripheral blood post-therapy as well as many research applications that require mutation detection at low levels. This technique will also help investigators to identify samples that contain their mutation of interest from wild type samples saving time and money. In continuing to further investigate low level mutation detection and genotyping in variable genomic conditions, testing of clinical samples is currently being planned.

Figure 4: Detection of 0.1% Mutant Allele of the BRAF v600e Target

- Heterozygous
- 10% Mutant Allele Fraction
- 1% Mutant Allele Fraction
- 0.1% Mutant Allele Fraction
- 100% Wildtype



CONTACT INFORMATION

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