Deterministic Design and Implementation of Single Nucleotide Variant Genotyping by High Resolution Melting

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Introduction/Background

PCR and high resolution melting is potentially the simplest and fastest method for genotyping single nucleotide variants (SNVs). PCR primer design is a first and crucial step of assay development. Overwhelming amount of available software with numerous criteria commonly yield disappointing primer pairs requiring re-design. We propose a simple method for both primer design and optimal PCR conditions development so that the process works well the first time.

Materials/Methods

The 52 forensic SNVs were selected as the target for our study. PCR primers were designed by Adam™ software (Figure 1). Universal PCR protocol for simultaneous amplification of all 52 SNVs was developed, consisting of a 2-step touch down (Figure 5.).

Results

Using Adam™ primer design and universal protocol, we successfully amplified and produced melting curves for all 52 SNVs. In 7 out of 52 cases, the melting curves of the wild-type (WT) and mutant homozygote (HOM) SNVs were indistinguishable due to sequence thermodynamics. All 7 cases were correctly predicted by uMELT (https://dna.utah.edu/umelt/umelt.html).The reliability of this prediction allows us to incorporate automatically designed snapback genotyping probes on the 5' end of one of the PCR primers in order to type 100% of WTs and HOMs (Figure 3, 4).

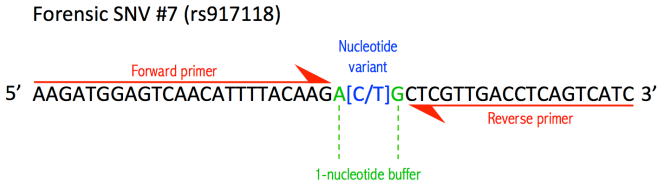


Figure 1. A representative example of primer pair design using our software. Adam™ places the 3' end of both reverse and forward primers next to the SNV with a 1-nucleotide buffer. Subsequent nucleotides are then added to the 5' end of the primers until the predicted melting temperature (Tm) reaches 60˚C.

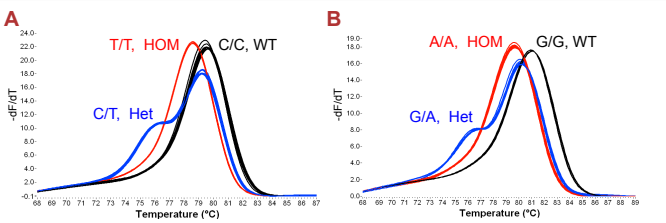


Figure 2. Derivative melting curves for SNV #7 (A) and #53 (B) genotyping. Twenty four individuals of varying ratios of WT, Het and HOM genotypes were analyzed.

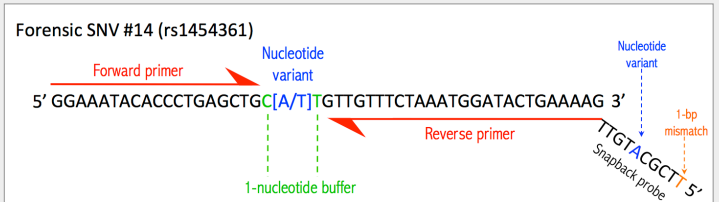


Figure 3. Snapback probe assay design for differentiation of WTs and HOMs where these produce identical melting curves. A snapback (in this case, reverse) primer includes a 5' tail complementary to the region surrounding the SNV.

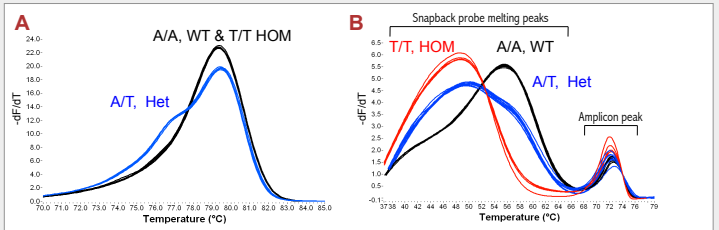
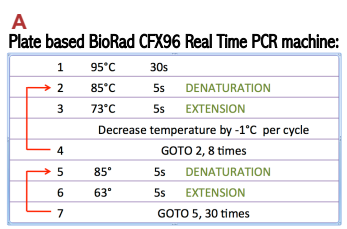


Figure 4. Genotyping of SNV #14 with snapback primers. Derivative melting curves of WT and HOM amplicons (A) are indistinguishable, but the snapback probe (B) allows a clear differentiation of individual genotypes of the A>T variant.



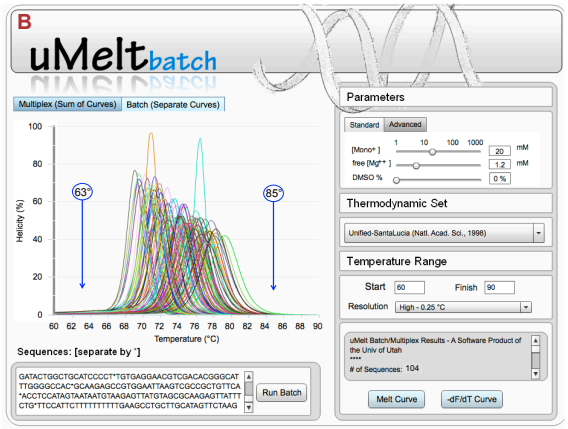


Figure 5. Universal touch down PCR protocol (A). Choice of denaturation temperature (85°C) was determined by the Tm of the 52 amplicons to prevent the amplification of high Tm products. The annealing/extension temperature (73°C, gradually decreasing to 63°C) was based on the primer Tms that varied between 58.42 and 61.14˚C. Batch melt prediction (B) of all 52 PCR products considering both the WT and HOM scenario. Calculated using uMELT batch software (https://www.dna.utah.edu/umelt/umb.php).

Conclusions

A simplified primer design algorithm provided clear PCR results in 100% of the targets tested when amplified according to the universally optimized PCR protocol. The algorithm did not discriminate against primers with sequence runs or repeat, or sequences producing primer-dimers. This method reduces the complicated task of assay design to a user-friendly and effective algorithm requiring only knowledge of the target sequence and a basic thermodynamic calculation.

References:

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