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RESEARCH REPORT

Genotyping a Class 4 SNP by high resolution melt (HRM) using SYBR Green I

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Introduction

The use of high dye concentrations is thought to be advantageous for HRM (high resolution melt) since it is presumed to reduce the probability of dye redistribution effects during strand dissociation (according to the dye saturation model of Wittwer et al 2003). Because of this, focus has remained on the use of third generation dsDNA intercalating dyes such as LC Green®, SYTO®9 and EvaGreen™ as their low reaction toxicity allows them to be used at relatively high concentrations.

In deference to the saturation model, second generation dyes such as SYBR® Green I (SYBR) have largely been avoided for HRM due to the relatively low (non-saturating) concentrations of dye that must be used to avoid reaction inhibition (Liew et al 2004). In contradiction to this, recent publications have shown that SYBR can be used successfully for HRM using a Rotor-Gene 6000® analyzer (Price et al 2007; Pornprasert et al 2008), casting doubt on the validity of the dye saturation model. We therefore question the accepted dogma that restricts HRM methodology to only "saturating" dye concentrations.

In this report we compare the efficacy of SYBR dye under non-saturating conditions with "saturating" dyes in an especially demanding HRM application; the accurate genotyping of a Class 4 SNP (single nucleotide polymorphism). Of the possible single base pair polymorphisms, the A to T Class 4 SNP is the most challenging to resolve since homozygous genotypes differ least in their T_M (typically by only about 0.2°C). Currently, the Rotor-Gene 6000 is the only instrument with sufficient thermal precision to resolve Class 4 SNP homozygotes by HRM. To make this assessment easily repeatable by others, we provide full sequence information and used three standard commercial master mix chemistries, two of which contain different "saturating" dyes formulated for HRM and one containing a "non-saturating" concentration of SYBR dye (formulated for regular real-time amplification analysis).

Methods

We used a 101 bp synthetic A to T SNP assay, the primer and amplicon sequences of which are: forward primer; AAC TTG GCT TTA ATG GAC CTC CA: reverse primer; ACA TTC ATC CTT ACA TGG CAC CA: amplicon sequence; AAC TTG GCT TTA ATG GAC CTC CAA TTT TGA GTG TGC ACA AGC TAT [W]GA ACA CCA CGT AAG ACA TAA AAC GGC CAC ATA TGG TGC CAT GTA AGG ATG AAT GT, where W is the IUPAC code for an A to T substitution.

The following commercial master mixes were used with recommended concentrations of template (25 ng.µL^{-1}) and primer (300 nM); SYBR® Green PCR Master Mix (Applied Biosystems, USA), SensiMix HRM™ with EvaGreen™ dye (Quantace, UK), and LightScanner® Master Mix with LC Green Plus® dye (Idaho Technologies, USA).

The A to T SNP assay was run on a Rotor-Gene 6000 under the following cycling conditions: initial denaturation; 95°C for 10 min; 40x two-step cycles of 95°C for 10 sec and 60°C for 15 sec. At the completion of cycling, HRM was run using 0.1°C increments with a 2 sec hold at each increment from 75°C to 90°C. Fluorescent signal was acquired on the dedicated HRM channel (460±15 nm excitation; 510±5 nm detection). Raw HRM data was analyzed by isolating and normalizing raw fluorescence data over the melt domain followed by a difference plot normalized to AA genotypes. Genotypes were auto-called by the Rotor-Gene HRM software.

Results

The three genotypes (homozygous AA, TT, and heterozygous AT) were clearly identified using each of the master mixes (Figure 1). Subtraction plots exhibited minimal spread between replicates for each chemistry tested. Rotor-Gene HRM software was able to automatically assign all genotypes with >97% confidence, irrespective of the chemistry used. Differences in the shape of the melt curve obtained for each genotype were observed as well as in the absolute temperature of melting between master mix chemistries. These differences are presumably due to the variations in ionic formulation and did not obscure results.

Conclusion

The data presented here clearly demonstrates that SYBR can be used at non-saturating concentrations, including for demanding HRM applications such as genotyping and auto-calling class 4 SNP homozygotes.

References

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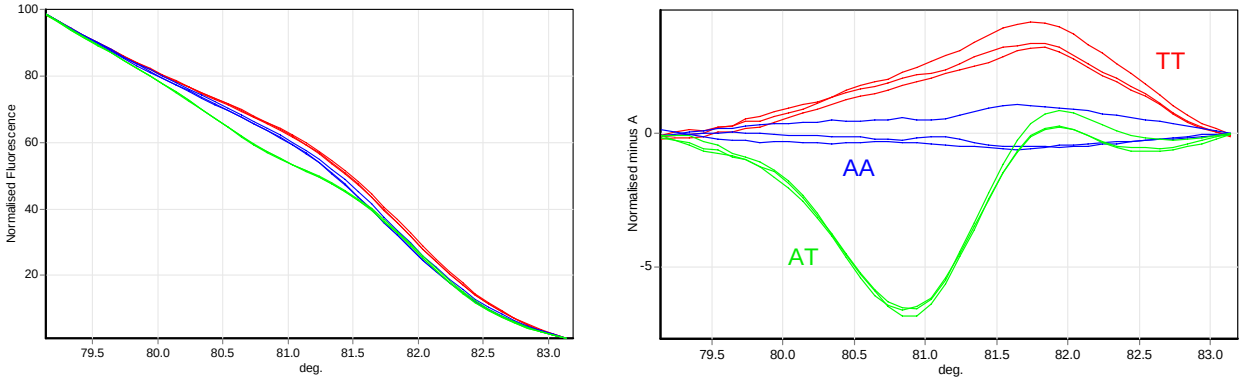
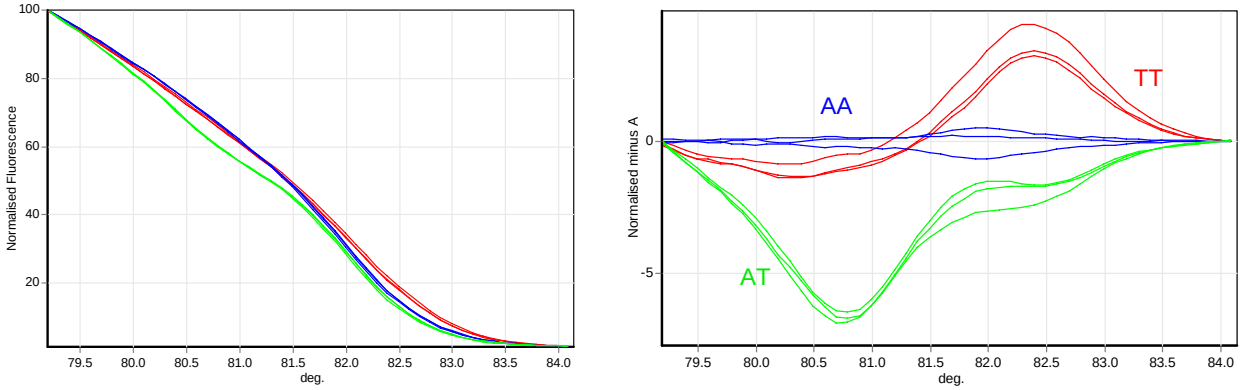
A SYBR® Green 1 (SYBR® Green PCR Master Mix; Applied Biosystems)



B EvaGreen™ (SensiMix HRM™; Quantace)



C LCGreen Plus® (LightScanner® Master Mix; Idaho Technology)

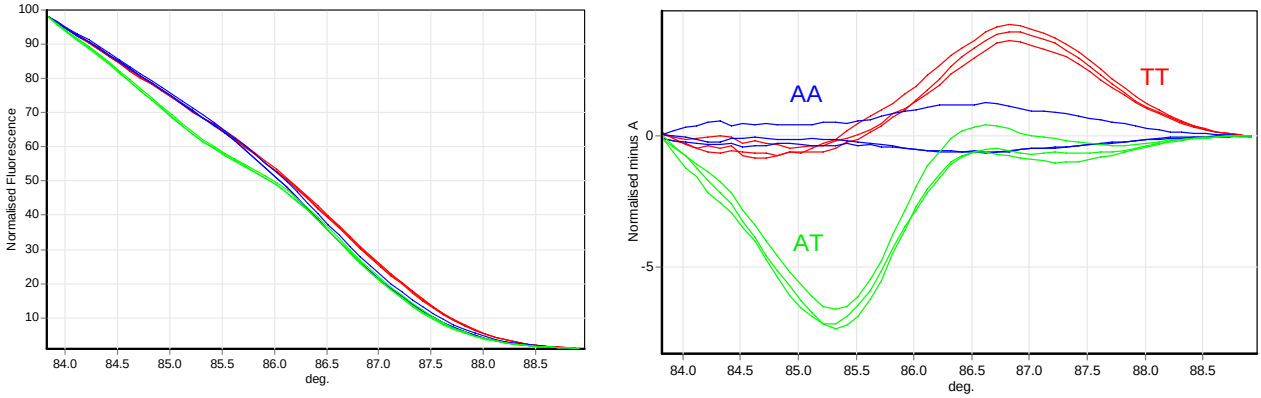


Figure 1: Chemistry comparison for Class 4 (A to T) SNP genotyping by HRM analysis on the Rotor-Gene 6000
Normalized HRM melt curves (left) and difference plots normalized to AA genotypes (right) of an A to T SNP performed with A: SYBR® Green PCR Master Mix (Applied Biosystems (USA), B: SensiMix HRM™ from Quantace (UK), and C: LightScanner® Master Mix from Idaho Technologies (USA). Three replicates of each genotype (A = blue, T = red and AT = green) were run with each master mix. All genotypes were clearly distinguished by each alternative master mix.