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BACKGROUND

High resolution melt (HRM™) analysis is a rapidly developing technique for mutation detection based on the temperature dissociation characteristics of DNA. Development of the technique has relied on the continued evolution of fluorescent binding dyes. These “Third-Generation” dyes include LCGreen⁺, SYTO®9 and EvaGreen™. Previous comparative experiments of these dyes with SYBR® Green I identified the importance of saturating dye concentrations in reducing dye redistribution. It has been postulated that dye redistribution leads to a decrease in sensitivity and therefore reduced confidence in mutation calling. We have shown that LCGreen⁺, SYTO9, EvaGreen and SYBR Green I are not saturating at concentrations non-inhibitory for PCR. EvaGreen, although non-saturating, successfully detects class IV mutations. These results lead to the conclusion that dye-chemistries and not saturation *per se* are important principles that need to be addressed for successful HRM analysis. All experiments were carried out on a Corbett Life Science Rotor-Gene™ 6000 HRM system using SensiMix HRM.

1. PCR INHIBITION

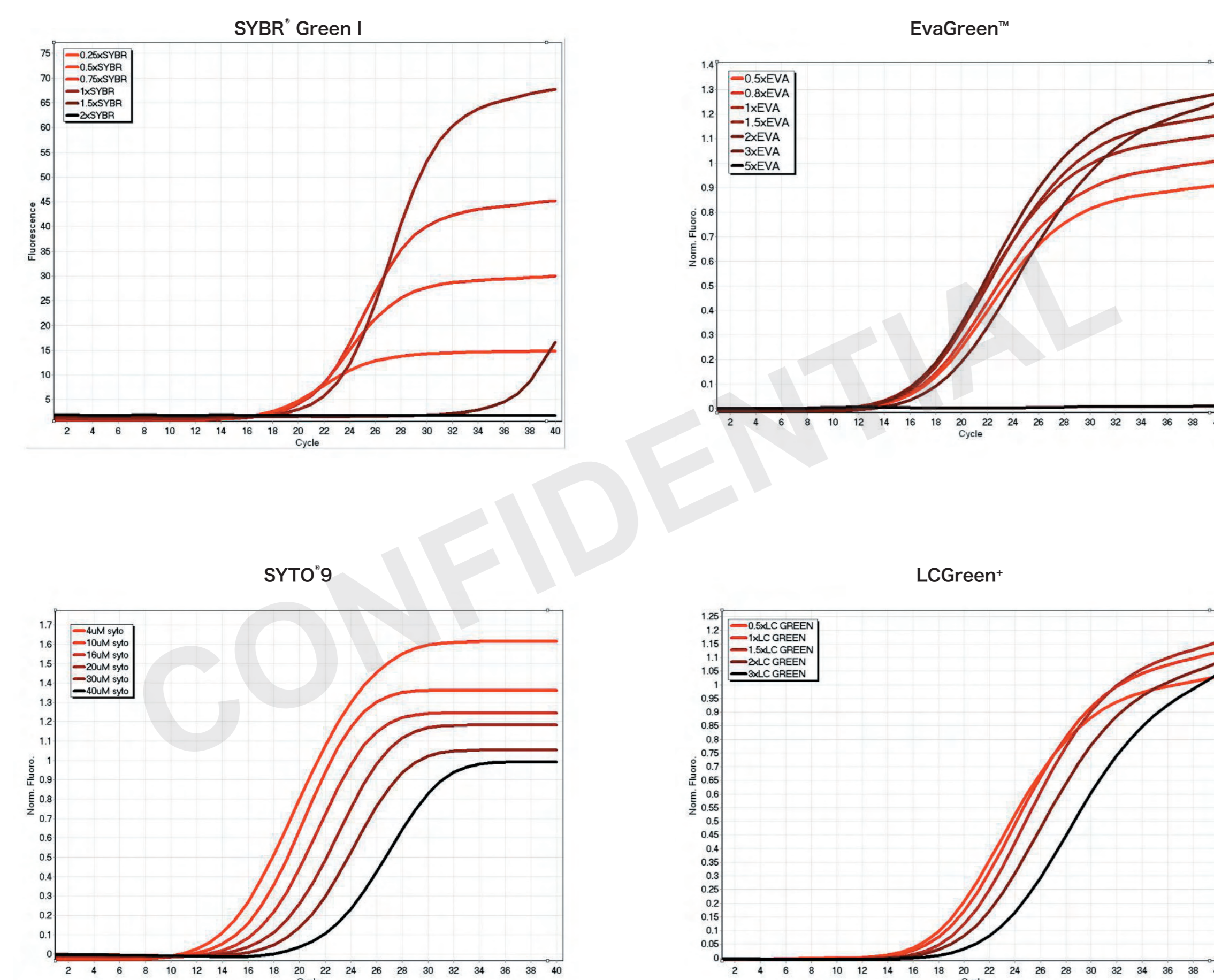


Figure 1. Inhibition of PCR by SYBR Green I, EvaGreen, SYTO9 and LCGreen⁺.

PCR was carried out using the above dyes at concentrations shown in each legend. Reactions were carried out in triplicate on plasmid-derived template DNA. Results demonstrate complete inhibition of PCR by SYBR Green I and EvaGreen. SYTO9 and LCGreen⁺ did not completely inhibit PCR at the concentrations tested. Due to the supplied concentration of LCGreen⁺, higher concentrations could not be tested for complete inhibition.

2. DYE SATURATION

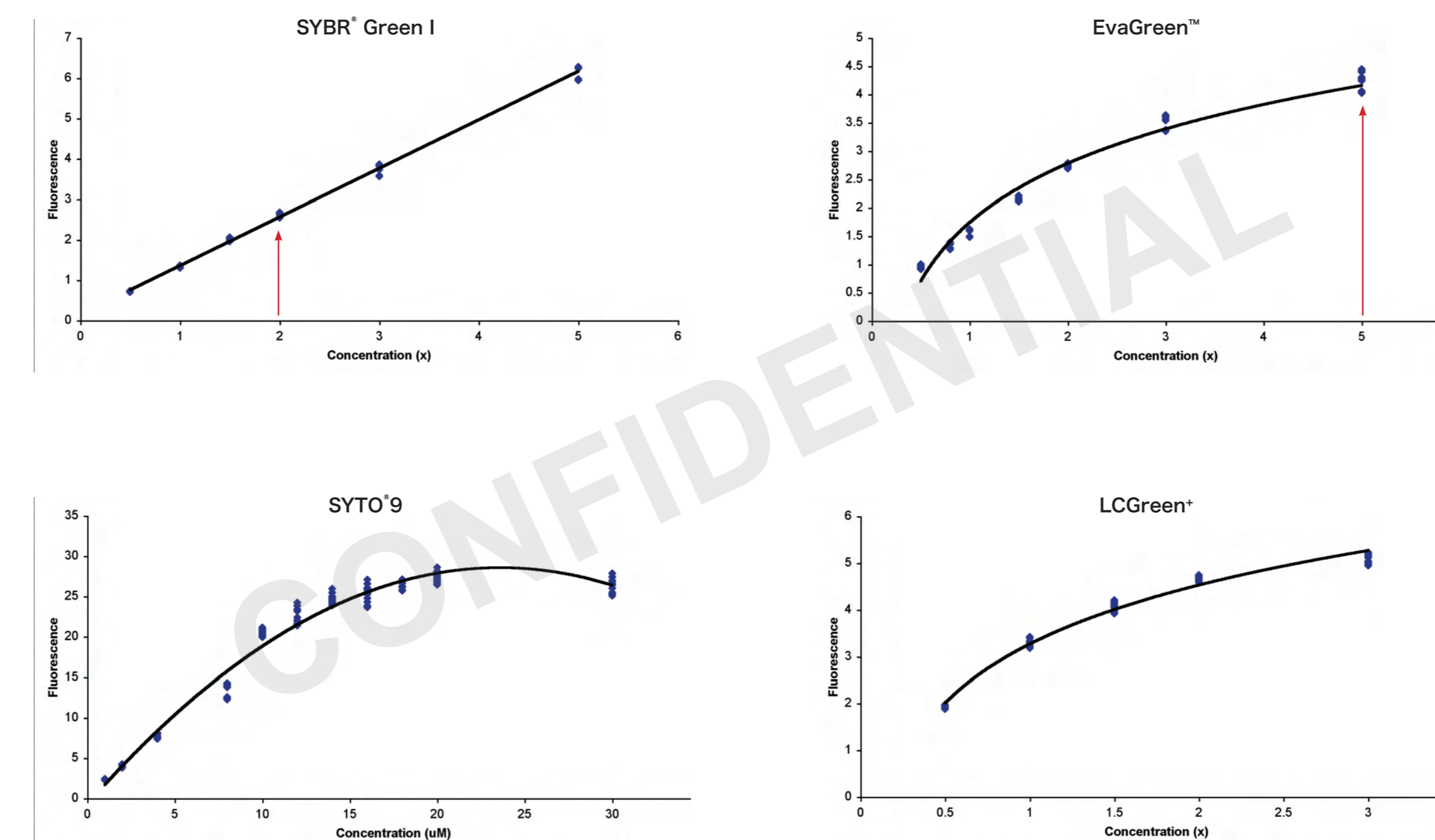


Figure 2. Saturation analysis of SYBR Green I, EvaGreen, SYTO9 and LCGreen⁺

Saturation analysis was carried out on a 100bp PCR product. Fluorescence was measured following incubation in SensiMix HRM with the above dyes. Only SYTO9 was shown to saturate the DNA at concentrations which do not completely inhibit PCR (Figure 1). EvaGreen and SYBR Green I were found to be non-saturating at non-inhibitory concentrations. Due to the supplied concentration of LCGreen⁺, higher concentrations could not be tested for saturation.

Arrow (↑) indicates the concentration at which complete PCR inhibition occurs.

Analysis was repeated on a 200bp PCR product and a no DNA control (data not shown).

3. DYE REDISTRIBUTION

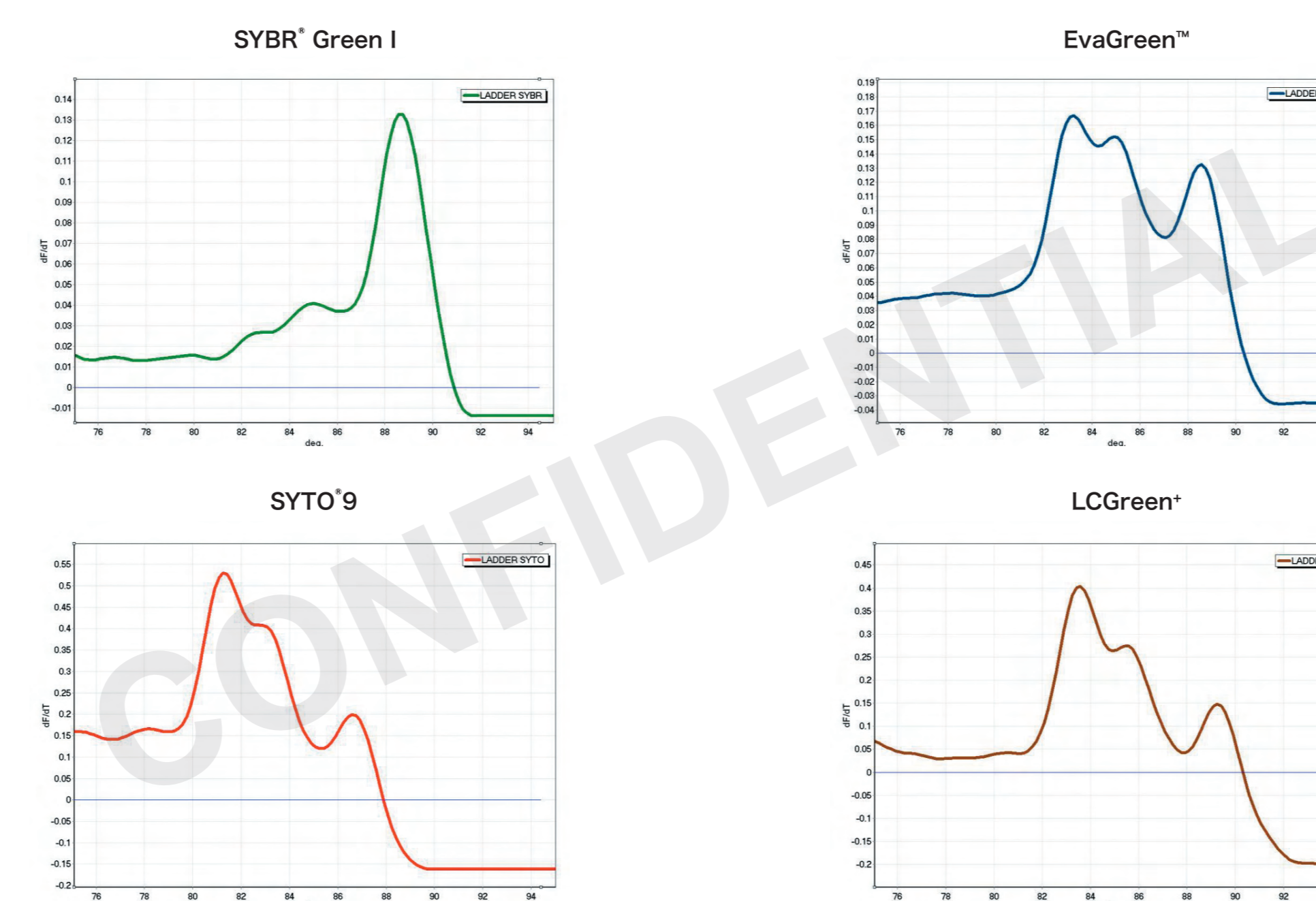


Figure 3. Dye redistribution analysis.

Derivative melting curves of a ladder comprising of 75bp, 200bp, 1.2kb and 2.5kb fragments. Comparison with individual melts (data not shown) demonstrates that SYBR Green I redistributes from low to high-temperature melting species. SYTO9, EvaGreen and LCGreen⁺ display minimal redistribution. All of the dyes were used at concentrations shown to be non-saturating and therefore saturation of the DNA is not required to prevent redistribution.

4. SINGLE NUCLEOTIDE POLYMORPHISM DETECTION

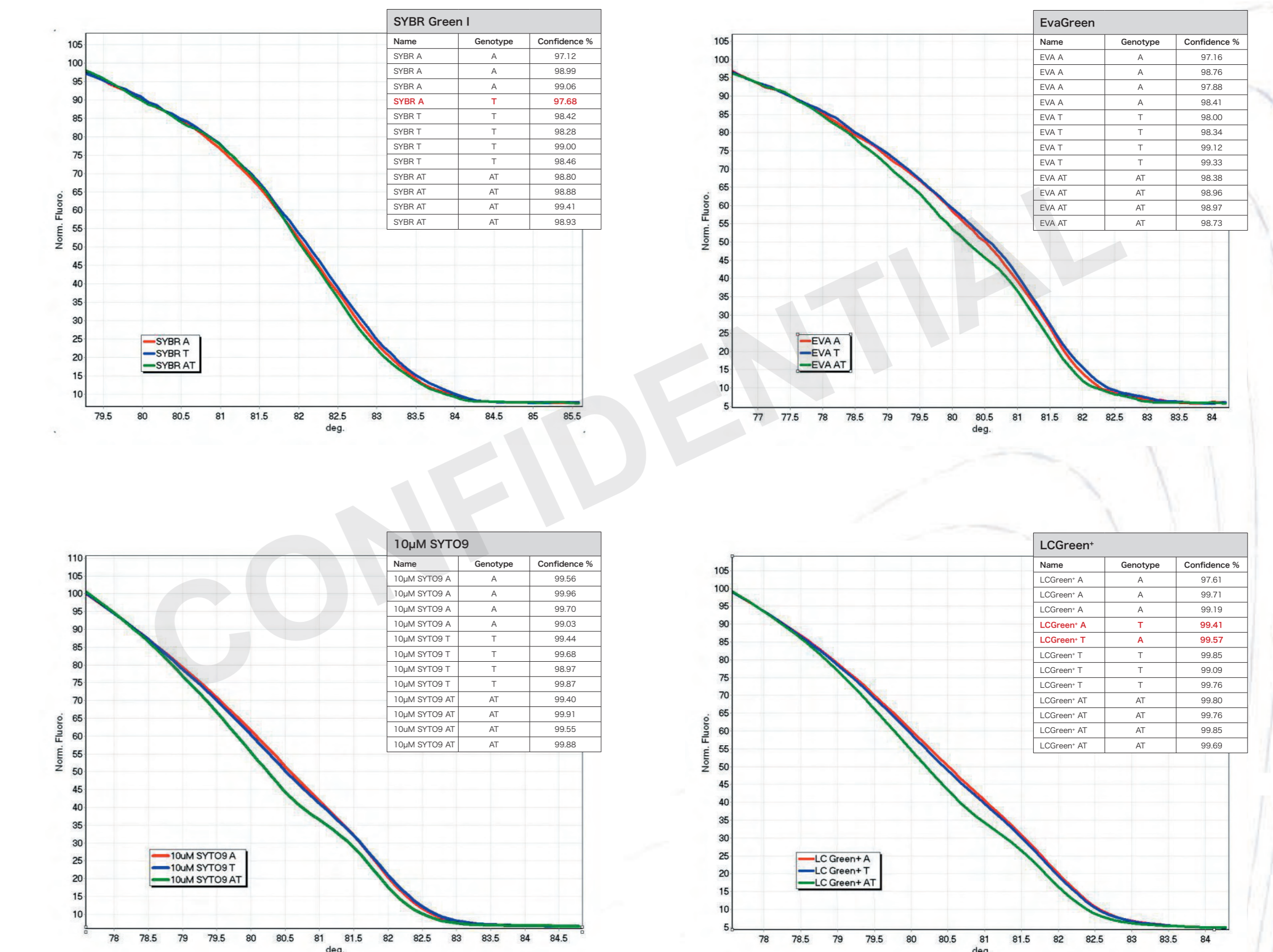


Figure 4. Class IV mutation detection

Class IV Single Nucleotide Polymorphisms (SNPs) are the most difficult to differentiate based on the melt profile. The three different genotypes of an A>T SNP were analyzed by HRM. Templates were plasmid derived. The dyes were used at concentrations non-inhibitory to PCR and therefore non-saturating.

The graphs illustrate normalised melt data from four replicates. Confidence values were calculated using the Rotor-Gene software.

CONCLUSION

1. SYBR Green I and EvaGreen completely inhibit PCR under non-saturating conditions. In contrast, SYTO9 and LCGreen⁺ are not completely inhibitory to PCR at the concentrations tested.
2. With the exception of SYTO9, none of the dyes tested were found to saturate DNA.
3. SYBR Green I displays the highest tendency to redistribute during DNA melting
4. The data presented demonstrates that saturation of the DNA is not a prerequisite for accurate mutation detection using HRM analysis.

Acknowledgments

We are grateful to Corbett Life Science for their continued support and Andrew Stevens for graphic assistance.

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