

# Factor V Leiden (G1691A) and prothrombin (G20210A) genotyping by High-Resolution Melt Analysis (HRM) and Syto 9 fluorescent Dye on the Rotor-Gene 6000 HRM device

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## INTRODUCTION

Several methods have been developed to detect common single point mutations in the factor V and prothrombin genes that are risk factors for thrombophilia. Most are based on PCR followed by restriction enzyme digestion and electrophoresis (RFLP), but gel analysis has certain limitations. Other detection methods based on real-time PCR have been described. The newly developed methods offer several advantages over conventional methods. However, they are limited by the requirement that the sequence variants need to be in the same melting domain as the probe, and one or more expensive labeled probe are required. The goal of our study was to develop a close-tube screening method for these point mutations that did not require labeled probes.

## METHODS

In this study we developed and evaluated a method based on High Resolution-Melt analysis (HRM). This mutation scanning technique uses a saturating double-stranded DNA dye, Syto 9 and the High-Resolution Melter Rotor-Gene 6000 HRM. Mutations in PCR product are detected by changes in the shape of the melting curve compared to a reference sample. One hundred subjects were screened for the two mutations, and the results were compared to these obtained by multiplex real-time PCR with Fluorescent Resonance Energy Transfer (FRET) probes.

## RESULTS

Figure 1 (A, B) and figure 2 (C, D) show typical differential normalized melt curves and plots for wild-type, heterozygous and homozygous samples for the factor V Leiden and the prothrombin mutations. High Resolution Melting assay (HRM) and FRET-based screening of 100 samples gave identical results: 25 samples were heterozygous and 5 homozygous for the factor V Leiden mutation; while 10 samples were heterozygous and 2 homozygous for the prothrombin mutation (Table 1).

Table 1: Comparison of factor V Leiden and prothrombin genotyping data obtained by High Resolution Melting analysis (HRM) and FRET assay

	Factor V Leiden (n = 100)	Prothrombin G20210A (n = 100)
Wild-type	70	88
Heterozygous mutation	25	10
Homozygous mutation	5	2
N° of subjects compared (HRM vs FRET)	100	100
% concordance (HRM vs FRET)	100	100

## REFERENCES

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## CONCLUSION

This study shows that High-Resolution Melting analysis (HRM) clearly discriminated among wild-type, heterozygous, and homozygous status for the two mutations, and the results were in full agreement with those of the FRET assay. This assay is highly sensitive and accurate. It is also rapid, safe, and less costly than probe-based genotyping assays and, unlike conventional methods, it is a closed assay system that requires only PCR unlabeled primers and saturating double-stranded DNA dye, Syto 9.

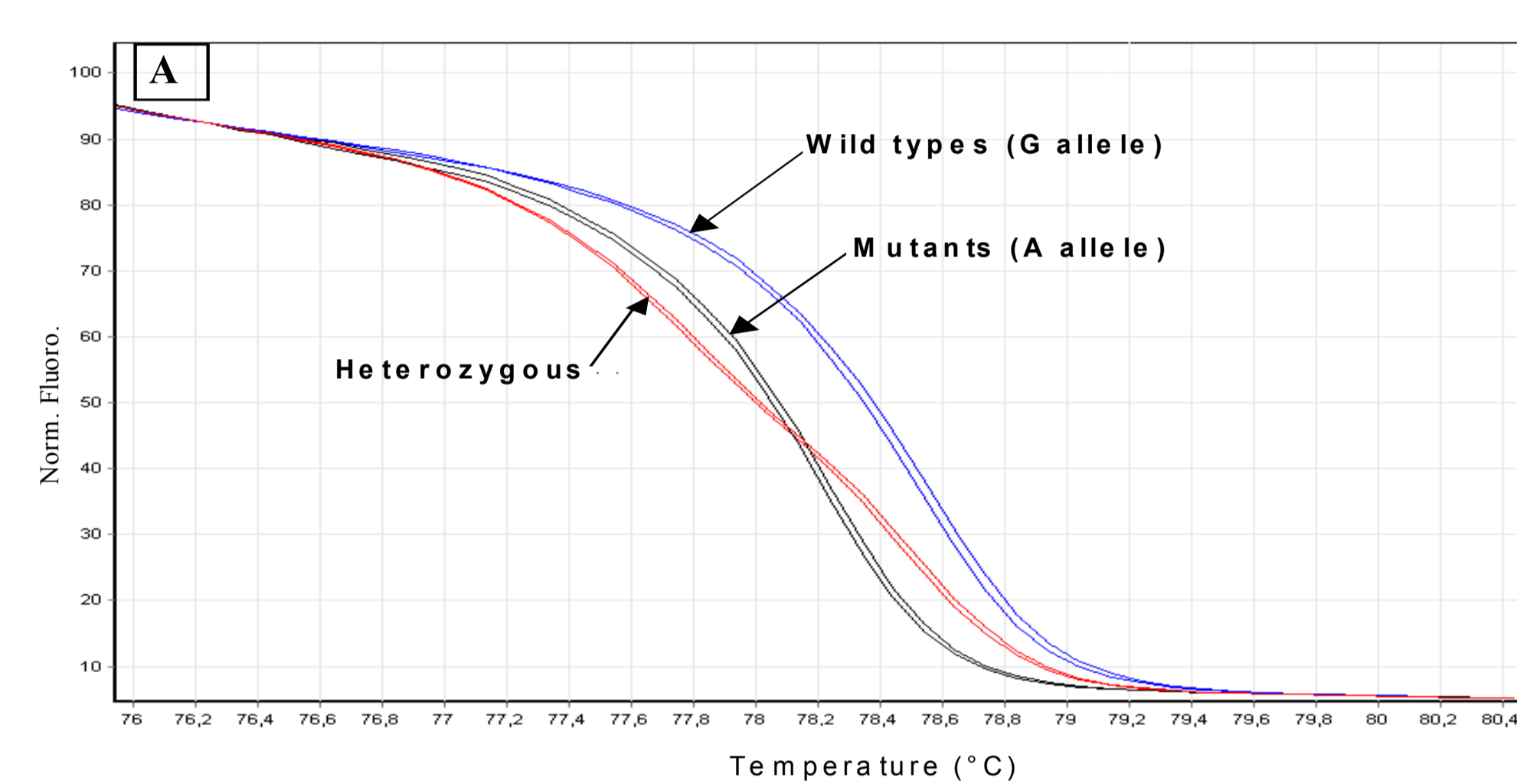


Figure 1 A: Normalized melt curves of the G1691A factor V mutation. Six samples: 2 wild type, 2 heterozygous and 2 mutant. The melt profiles shown are: GG wild type, blue (right); AA homozygous, dark (middle) and the AG heterozygous, red (left).

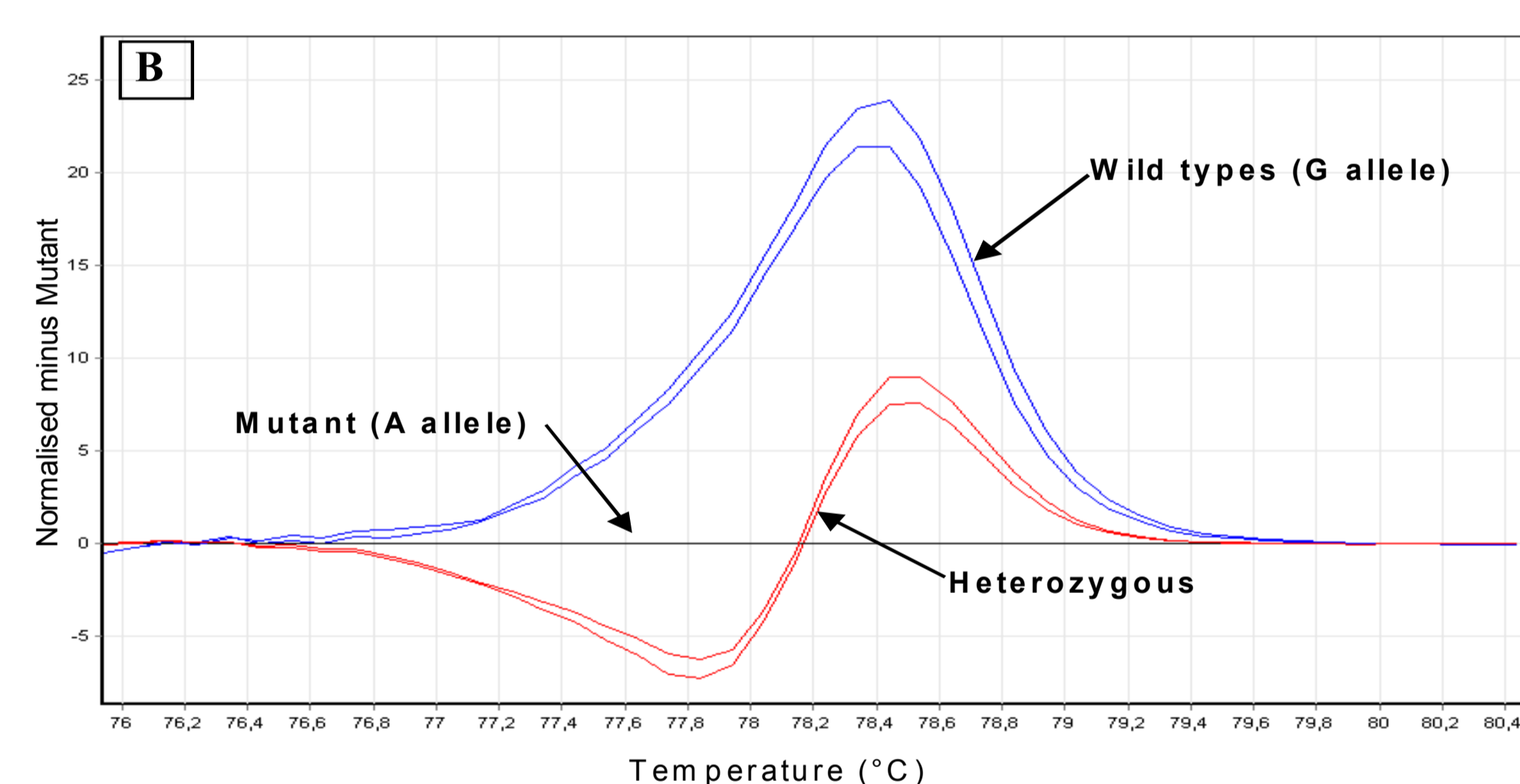


Figure 1 B: A difference plots of the G1691A factor V mutation. Six samples: 2 wild type controls (blue) and 2 heterozygous controls (red). All samples were compared to the median mutant controls (2 samples, dark).

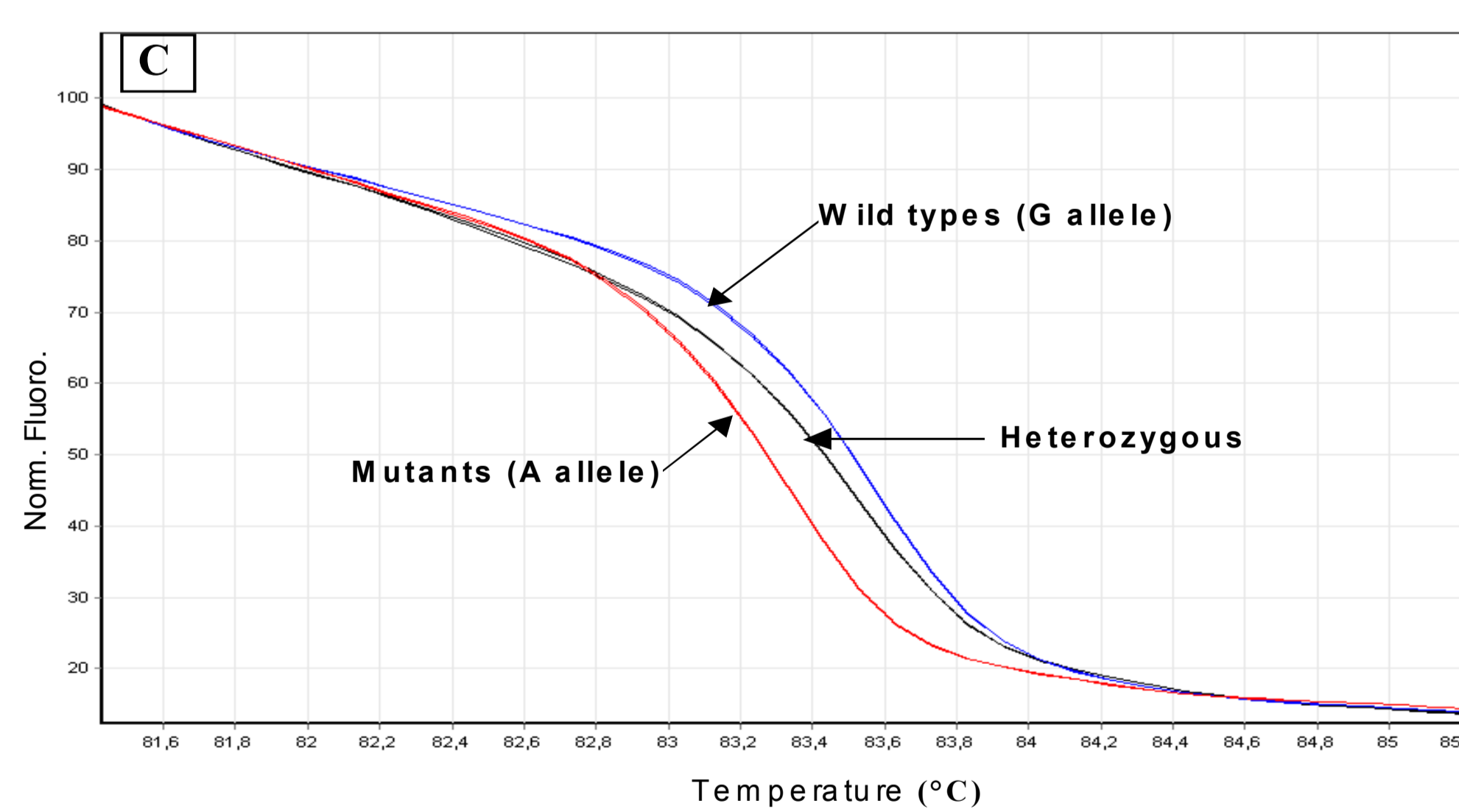


Figure 2 C: Normalized melt curves of the G20210A prothrombin mutation. Six samples: 2 wild type, 2 heterozygous and 2 mutant. The melt profiles shown are: GG wild type, blue (right); AA homozygous, red (left) and the AG heterozygous, dark (middle).

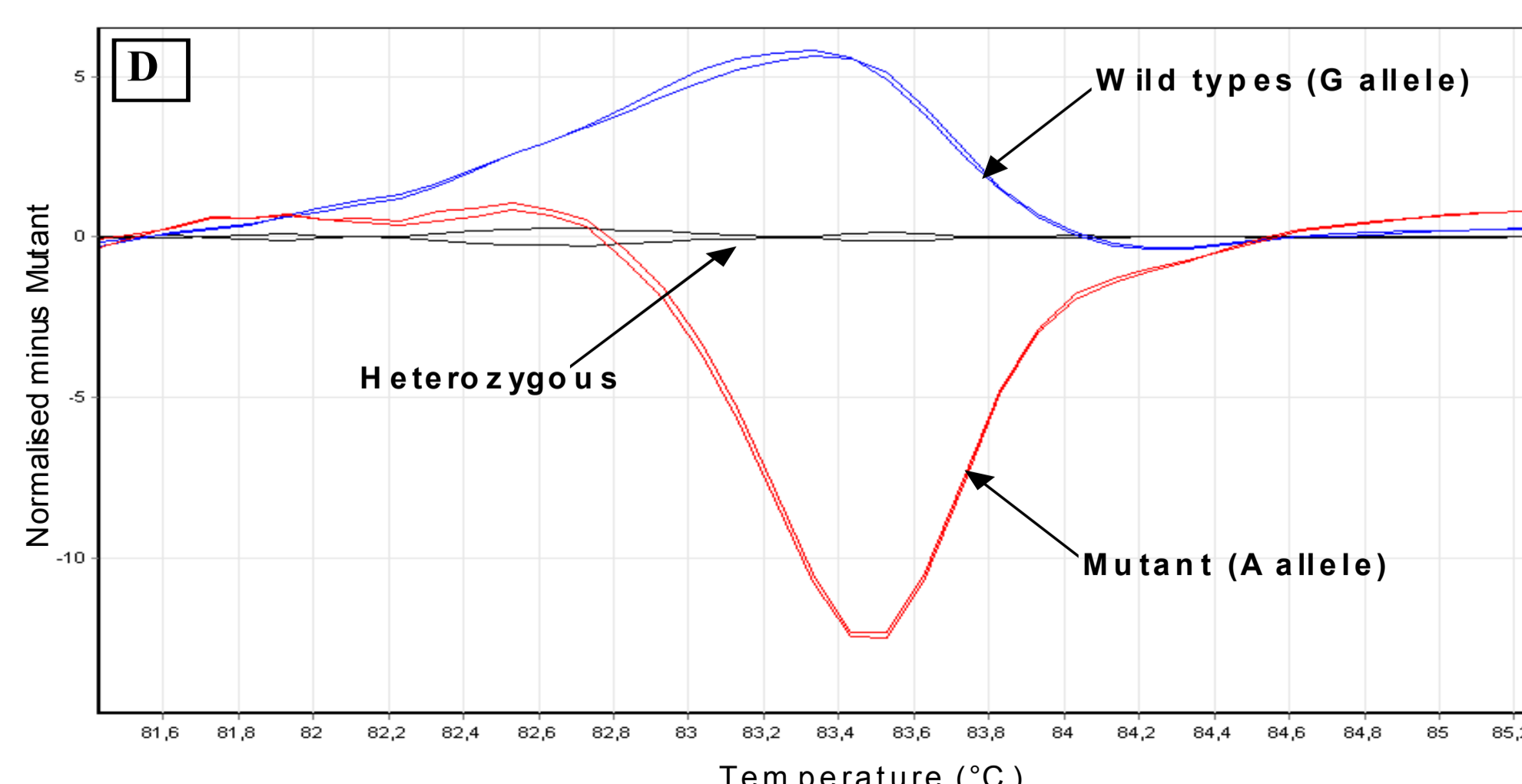


Figure 2 D: A difference plots of G20210A factor II mutation. Six samples: 2 wild-type controls (blue) and 2 mutant controls (red). All samples were compared to the median heterozygous controls (2 samples, dark).