# Multiplex Solution Genotyping Without Probes of the Factor V Leiden (G1691A), Prothrombin (G20210A), and MTHFR (C677T and A1298C) Mutations in One Tube by Single Color High-Resolution Melting Analysis.


University Health Care

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## ABSTRACT


**Background:** Venous and arterial thrombotic diseases are serious health concerns with well established links to the Factor V Leiden (G1691A), Prothrombin (G20210A), MTHFR (C677T) and MTHFR (A1298C) mutations. The likelihood of developing disease is increased with the presence of mutations in multiple clotting factor genes.

**Methods:** Four amplicons with non-overlapping melting temperature windows were created by varying the amplicon lengths from 40 base pairs to 120 base pairs and by using primers with G/C rich tails. Genotyping of each locus in the same closed-tube was based on the shape and position of the high-resolution melting transition. Heteroduplex melting analysis with LCGreen eliminated any need for probes.

**Results:** The Tm windows for each gene were separated by at least 1°C with the wild type melting temperatures of 76.0°C for Factor V Leiden, 88.5°C for Prothrombin, 84.5°C for MTHFR C677, and 80.5°C for MTHFR A1298. Within each window, the wild type, mutant, and heterozygous genotypes for each mutation, were resolvable. Concordant results between conventional LightCycler analysis and high-resolution melting were obtained on 120 blinded clinical samples.

**Conclusions:** Genotyping by amplicon melting is a rapid, closed-tube method for genotyping without probes that can be multiplexed. The method was successfully applied to the four most common clotting factor mutations in a single tube. The simplicity of the method allows rapid development of low-cost assays and does not require any arrays or complex instrumentation.


## INTRODUCTION

Venous and arterial thrombotic disease are serious health concerns throughout the world. Venous thrombosis is a multifactorial disease caused by the interaction of lifestyle and genetic factors. Genetic factors include well established links between the Factor V Leiden (G1691A) and Prothrombin (G20210A) mutations and increased risk of thromboembolic disease and coronary artery disease. Two other mutations have been linked to coronary artery and venous thrombosis disease through their effect on homocysteine metabolism. The mutations occur in the methylenetetrahydrofolate reductase (MTHFR) gene. The two observed mutations are (C677T) and (A1298C) which both cause hyperhomocysteinaemia.

Analytical DNA assays for the identification of these clotting factor mutations are broadly used in the clinical laboratory. Commercial and home-brew assays for these mutations are most commonly tested for by Fluorescence Resonance Energy Transfer (FRET) hybridization probes which are expensive.

As demand grows for these assays, so does the need for rapid, reliable, and cost effective methods of detection.

## MATERIALS/METHODS

**Samples:** 120 clinical Factor V Leiden, Prothrombin, MTHFR 677 and 1298 whole blood samples were collected in $K_3$EDTA, Na-Heparin, and CPD tubes and stored at 4°C. Samples were deidentified according to HIPPA regulations.

**Nucleic Acid Extraction:** MagNA Pure LC DNA Isolation Kit I (Roche)

**Nucleic Acid Amplification:**

- LightCycler (Roche)
- LightCycler FastStart DNA Master HybProbe Kit (Roche)
  - 20 µL reaction
    - 1X Kit master mix
    - 3.5 mM $MgCl_2$
    - 0.1 to 0.5 µM of each primer
    - 1X LightCycler Green Plus (Idaho Technologies)
    - 1 U heat-labile UNG (Roche)
    - 4 µL of DNA.
  - Cycling Parameters
    - 95°C 15 minute enzyme activation
    - Amplification (45 cycles ~ 25 minutes)
      - 2 second denaturation at 95°C
      - 1 second anneal at 56°C
      - 1 second elongation/fluorescence acquisition at 72°C.

**Standard Testing of Clinical Samples:**

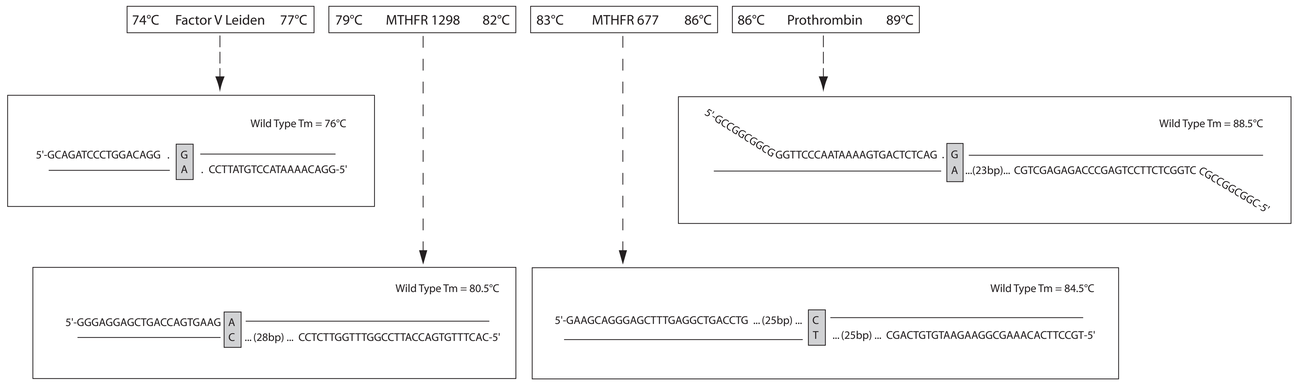
- Prothrombin genotypes were verified by the Factor II (Prothrombin) G20210A Kit (Roche)
- Factor V Leiden genotypes were verified by the Factor V Leiden Kit (Roche)
- MTHFR 677 and 1298 genotypes were verified by the ARUP assay

**High Resolution Melting Analysis:** HR-1 (Idaho Technology)

## MULTIPLEX PCR DESIGN

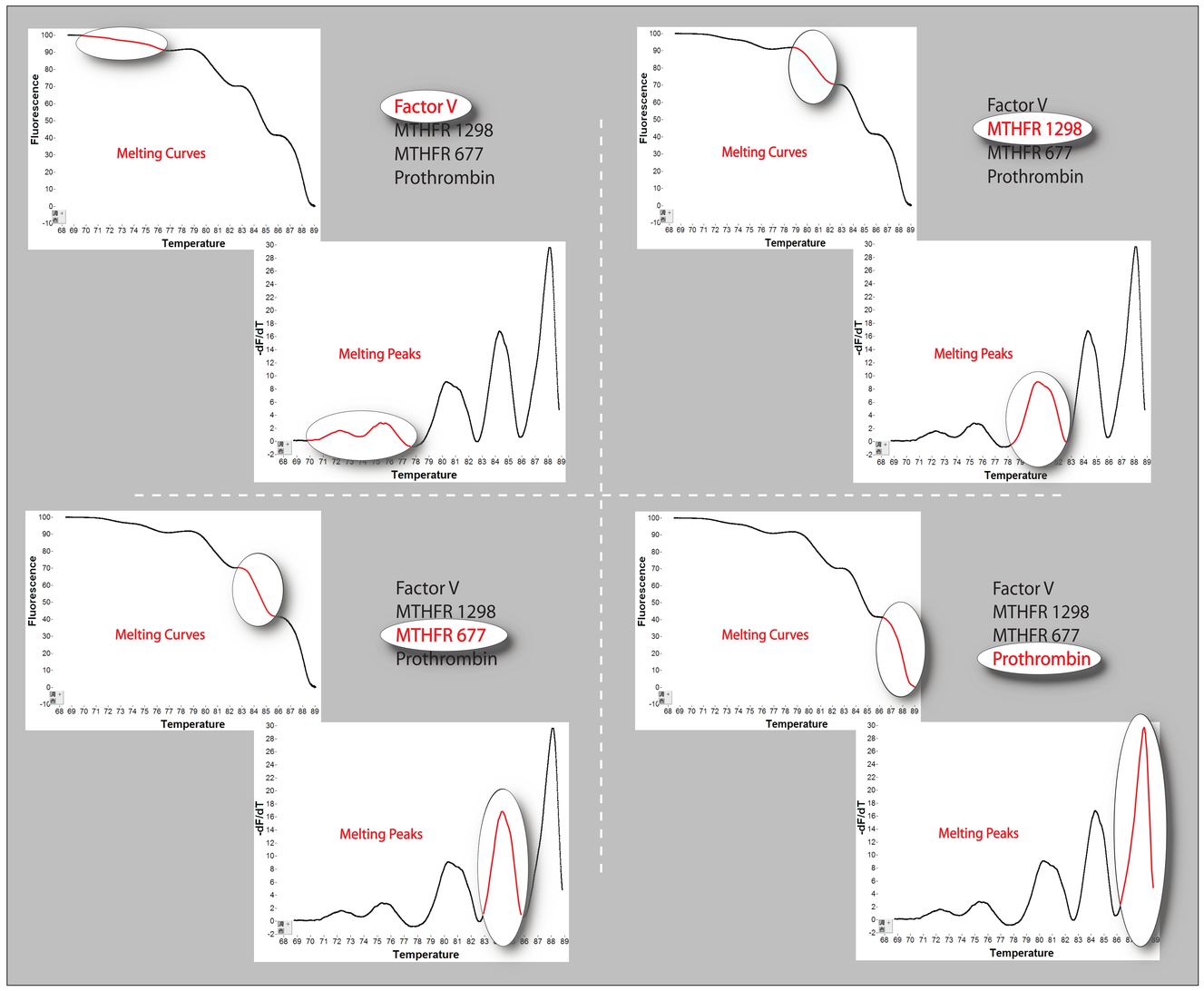
**Amplicon Design:** The quadriplex amplicons were designed to fit in distinct melting temperature windows. This was accomplished by adjusting the length and G/C content of the amplicon and use of tailing primers.

**Melting Curve Resolution:** PCR's within the quadriplex have different amplification efficiencies and the specific amplicons fluoresce differently. Primer concentrations were varied to optimize resolution and to adjust fluorescence signal strength for each temperature window.



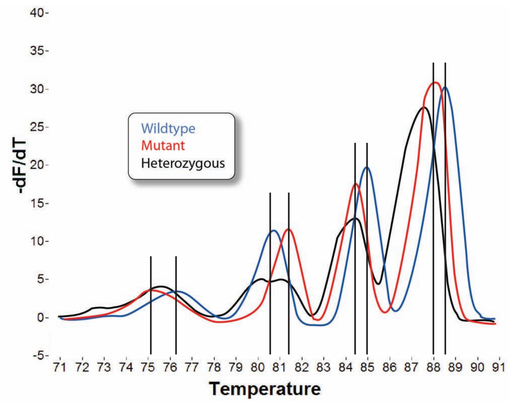

## HIGH RESOLUTION MELTING ANALYSIS

- Melting curves were generated using the HR-1 instrument (Idaho Technology) with a temperature transition rate of 0.1°C/s.
- A Super Heterozygous sample (Factor V Het, MTHFR 1298 and 677 Het, and Prothrombin Mut) was used as a control for Tm alignment.




## RESULTS

- **Genotyping:** Negative first derivative melting data for the blinded samples were compared to a super heterozygous control. Homozygous genotypes (wild type and mutant) were differentiated based on peak Tm while the heterozygous genotypes were differentiated based on derivative curve shape.




### Correlation to Standard Assay

| | Wildtype | Mutant | Heterozygous | Mutation Correlation |
|---|---|---|---|---|
| Factor V | 98/100 | 8/10 | 8/10 | 95% |
| MTHFR 1298 | 58/63 | 20/20 | 37/37 | 96% |
| MTHFR 677 | 56/57 | 25/27 | 35/36 | 97% |
| Prothrombin | 102/106 | 9/10 | 3/4 | 95% |
| Genotype Correlation | 96% | 93% | 95% | |

- The Factor V Leiden and Prothrombin quadriplex genotype results showed a 95% correlation. The MTHFR 1298 and 677 quadriplex genotype results showed a 96% and 97% correlation, respectively. The overall genotype correlation for the quadriplex was 96% for wildtype, 93% for mutants, and 95% for heterozygous samples.
- The majority of the incorrectly genotyped Factor V, MTHFR 1298 and 677, and Prothrombin samples were the result of peak Tm shifts which were observed in each Tm window of the quadriplex. It is suspected that salt concentration variations due to extraction effects and/or reation efficiencies cause the Tm windows to shift left or right.
- The five discordant MTHFR 1298 samples were called heterozygous by the quadriplex assay and wild type by the ARUP assay. This was most likely due to a mutation outside of the FRET probe region of the ARUP assay which was picked up by the quadriplex assay. These samples will be sequenced to determine the presence of other mutations in the amplicon region.

## CONCLUSIONS

- Using the most common clotting factor mutations as a model, the ease of genotyping by multiplexing PCR's through the shifting of amplicon Tm's has been demonstrated.
- Single color fluorescent dye multiplex PCR is a rapid and less expensive alternative to current methods of genotyping because multiple genotypes can be differentiated in a single reaction and no expensive labeled probes are used.
- This assay would benefit from the addition of a Tm reference control to adjust for Tm variations due to differing salt concentrations and reaction efficiencies.