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Extreme PCR Amplification on an In-line PCR Microfluidic System

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Introduction

Polymerase Chain Reaction (PCR) speed is not limited by chemical kinetic requirements but by instruments that are not able to temperature cycle rapidly. Extreme PCR, as recently introduced, is capable of pushing the reaction chemistry to its limits and provides high efficiency PCR amplification in < 2.5 minutes*. In addition to rapid amplification, analysis by small amplicon melting completes genotyping in 30 seconds*. We used a prototype microfluidic instrument to demonstrate sequential extreme PCR and genotyping by high resolution melting.

* Denotes change from abstract

Materials and Methods

Extreme PCR uses higher primer (2 mM each*) and polymerase (3.3 mM or 2.5 U/μL) concentrations than conventional rapid cycle[1] or legacy[2] PCR. Polymerases with fast extension rates, such as KlenTaq (Ab Peptides) used in these experiments, also speed PCR reactions. CleanAMP dNTPs (TriLink) were used for hot start PCR at a concentration of 200 μM each. A DNA concentration of 12 ng/μL was used for PCR reactions along with 3 mM $MgCl_2$, 500 μg/ml BSA and 50 mM Tris (pH 8.3). Oligonucleotide primers were designed for small amplicon genotyping of the Factor V Leiden mutation (1691G>A) with a product size of 43 bp.

F 5'- GCAGATCCCTGGACAGG
R 5'- CAAGGACAAAATACCTGTATTC

Reactions were thermocycled and melted using the Canon U.S. Life Sciences, Inc. prototype microfluidic genetic analyzer. Fluid flow was controlled within the microfluidic channels by monitoring the edge of a fluorescent dye, injected in-between each test solution, and utilized PID control of pumps at inlets and outlets (Figure 3). Reactions were run in a microfluidic chip containing eight discrete channels with embedded heaters., enabling rapid heat transfer. The thermocycling conditions were 95 °C for 1 minute (to activate the dNTPs) followed by 40 PCR cycles with programmed temperature transitions between 95 °C and 62 °C*. A cycle time of 1.75 seconds* gave a total PCR time of 70 seconds* (Figure 1). After completion of PCR, a melting ramp of 1.0 °C/s* was performed for genotyping which required an additional 30 seconds*. The melting data were analyzed using custom software: Melting Wizard: High-Resolution Melting Analysis (v5.11, University of Utah).

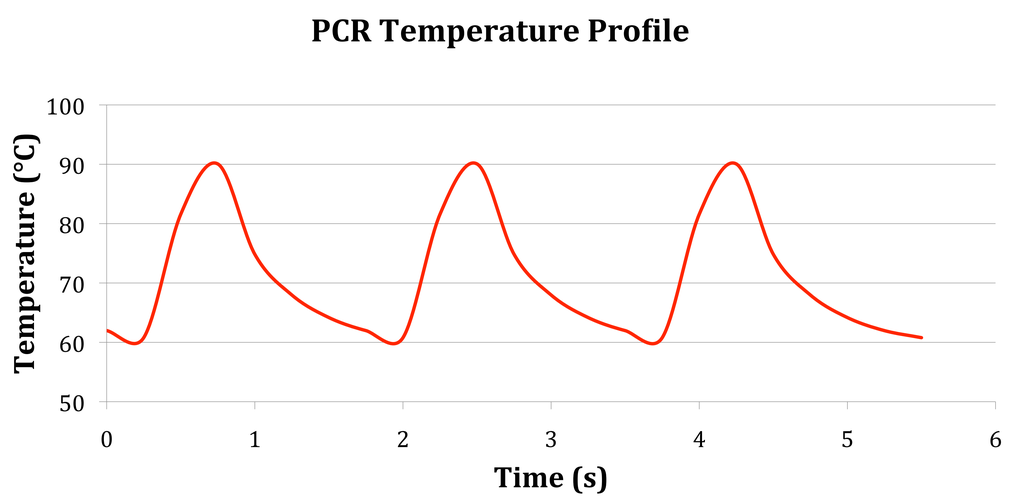

Figure 1: Representative PCR thermocycling temperature profile of one channel for 3 cycles with 1.75 seconds cycling times, which yields 40 PCR cycles in 70 seconds.

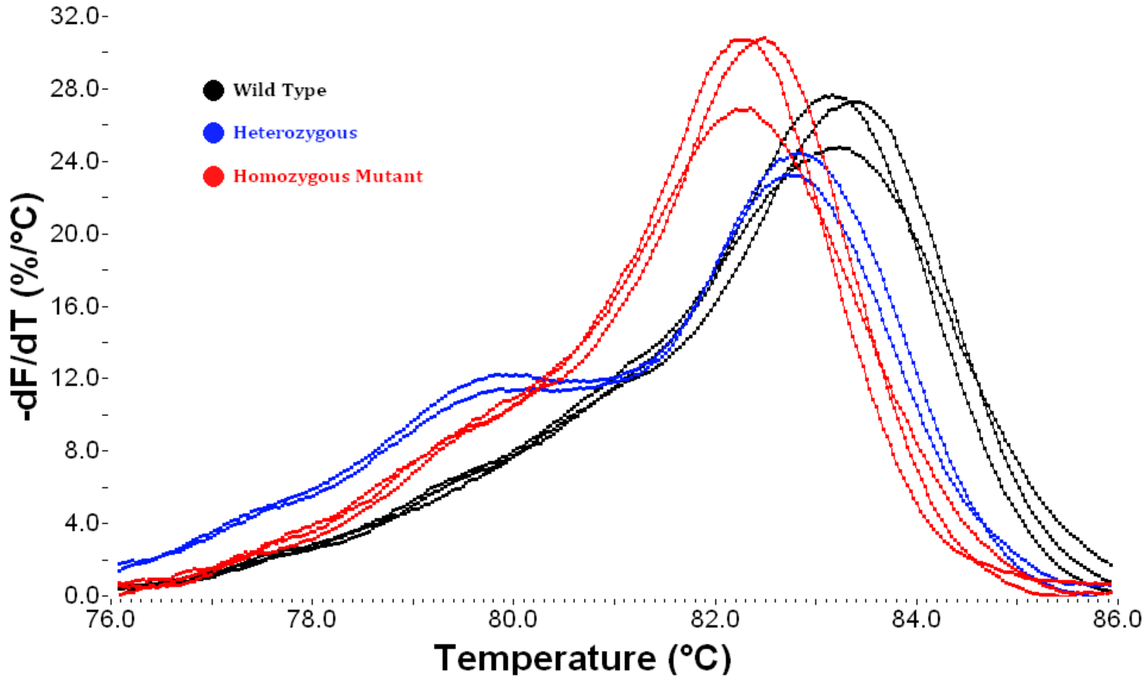

Figure 2: Genotyping of Factor V Leiden PCR amplicon. Genotypes were determined by derivative analysis of the 8 DNA amplicon melting curves using homogeneous background subtraction. The data is automatically clustered in the Melting Wizard software. The G>A mutation lowers the amplicon Tm which allows it to be distinguished from the wild type amplicon. The heterozygous amplicon is distinguished from the other two genotypes by a change in the shape of the melt curve due to heteroduplex complexes.

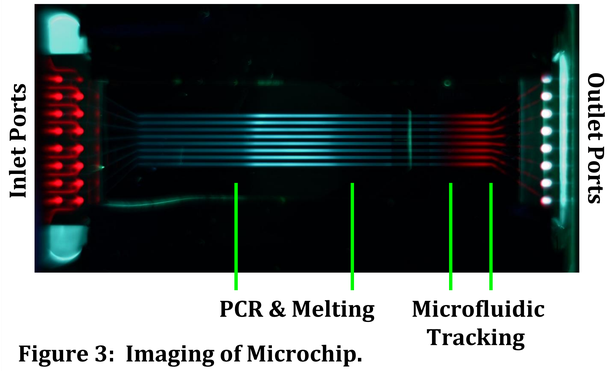

Figure 3: Imaging of Microchip.

Results

We demonstrated automated single nucleotide variant (SNV) genotyping of Factor V Leiden with PCR amplification in 2.2 minutes* and high resolution melting in 30 seconds* (Figure 2). Heterozygous PCR products were identified by the characteristic change in shape of the melting curve due to heteroduplex contributions. Homozygote PCR products were identified by their Tm differences.

Conclusions

This system demonstrates the automation of extreme PCR amplification followed by high resolution melting in < 3 minutes. Such extreme genotyping speed demonstrates the feasibility of genetic diagnosis "while you wait". Further work on optimizing PCR chemistry and instrument improvements will push the reaction time faster.

References

1 Wittwer CT, Garling DJ. Rapid cycle DNA amplification: time and temperature optimization.Biotechniques. 1991 Jan;10 (1):76-83.

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