Automation of a Genetic Assay With Real and Virtual Instruments

Integrating DNA Extraction, Amplification, High-Resolution Melting, and Analysis

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Overview

We have fully automated a genetic diagnostic assay for Factor V Leiden by combining an automated DNA extraction platform, a real-time PCR and melting instrument, and a `virtual instrument' implemented in software to analyze the results. After extraction and PCR in the presence of unlabeled oligonucleotide probes and saturating dsDNA dyes, heating and fluorescence acquisition and melting analysis are performed in the same closed tube. The virtual instrument automatically performs several AI tasks including data exclusion, feature recognition to optimize background removal and automatic clustering and classification to genotype the samples as wild-type, heterozygous, or homozygous for the Factor V Leiden mutation associated with a hereditary hypercoagulability disorder. For each 96-well plate, DNA was extracted and the plate prepared, rapid PCR performed, melting acquisitions obtained, mathematical analysis and genotyping in full concordance with independent validation finished in 295 minutes in comparison with 630 minutes without automation. The process is rapid, economical, non-destructive and contamination-free, since expensive fluorescently labeled probes and post-PCR preparations and separations are not required. Known polymorphisms and unknown variants can also be identified.

Introduction

Since the developments of rapid PCR and high-resolution melting techniques, the two remaining bottlenecks hindering the goal of high-throughput genotyping of genomic DNA have been extraction of DNA samples from blood and post-amplification analysis. Our objective is to demonstrate the possibility of integrating recent improvements in DNA extraction technology and mathematical algorithms into a real and virtual instrument hybrid to performs fully automated high-throughput genotyping.

Methods

By using multiple pipetting tools, the Protedyne Radius (Protedyne, Windsor, CT) system can extract 96 whole blood samples simultaneously and automatically which results in a decrease in processing time. The LightCycler 480 (LC480, Roche Diagnostics, Indianapolis, IN) allows for 96 samples to be processed at one time. In combination, these instruments enable us to link nucleic acid extraction with amplification and detection by high-resolution melting. 100 µL of whole blood is extracted and samples are supplemented with 100µL of elution buffer to maintain the correct reagent concentrations. The final elution volume is 100 µL. PCR set-up is performed on the Radius using 9 µL of pre-made mastermix and 1 µL of extracted DNA. For accuracy, 276 whole blood samples were extracted were amplified and detected using the Roche LC480 system for a Factor V Leiden unlabeled probe assay. DNA extracted on the Radius was evaluated for accuracy, precision, sensitivity, cross contamination, and sample type. All of the samples were examined for yield and purity by OD 260/280 ratios using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Samples were compared to previous results obtained from those assayed by the LightCycler using the Fac V Leiden Assay.

Three runs of 96 were used to evaluate contamination for the Radius system. Forty eight whole blood samples were set up with alternating water (no template control, NTC) samples to form a checkerboard. Raw data from the LC480 was exported to the MeltingWizard 3.0 analysis tool, implemented as a virtual instrument using LabVIEW 8.0 (National Instruments, Austin, TX). No template control samples were automatically excluded from further analysis by signal to noise estimation algorithms (Fig. 1-3). Feature recognition algorithms automatically identified the region corresponding to the melting of the probes (Fig. 4), and exponential background subtraction was performed. The normalized melting curves were automatically classified by genotype (Fig. 5) using hierarchical clustering algorithms adapted to high-throughput melting curve analysis. Melting temperatures were evaluated automatically using peak detection algorithms (Fig. 6). Between run and within run Tms were compared and genotypes validated in 100% concordance by the validation assay. Data from 15 96-well plates were analyzed in less than 15 minutes using this software

Results and Conclusions

The Protedyne Radius/LC480/MeltingWizard 3.0 system decreases processing time by 5.75 hours for 3x96 samples. It extracts genomic DNA from whole blood collected in different anticoagulant types without contamination, leading to accurate Tm and genotype results. Broken down into individual steps, the time reduction is:

Set-up	Extraction	Mastermix	PCR setup	Storage	Detection
15 min	80 min	30 min	80 min	105 min	45 min.

The virtual instrument accurately automates analysis and decreases processing time by 3.75 hours for 15 96 well plates. Screenshots from the key automation steps of the automated analysis are shown in the figures. Typical genotype and Tm results produced by the software are shown below. We conclude that clinical implementation of this system can significantly increase our sample throughput.

Sample	Genotype	# of Peaks	Tm_1	Tm_2	Δ Tm
A1	NTC/PoorSignal	0			
B1	WT	1	71.00		
A2	HET	2	66.06	71.00	4.94
C2	MUT	1	66.16		

Figures

Figure 1: Raw Data Before Automatic Data Exclusion

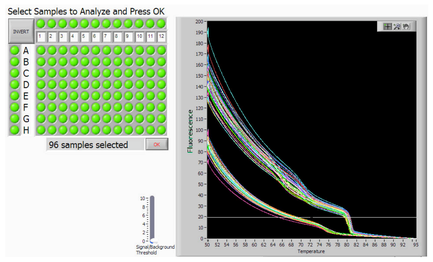

Figure 2: Raw Data After Automatic Data Exclusion

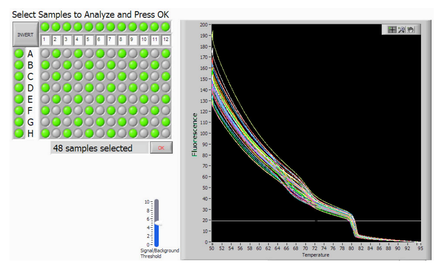

Figure 3: Data and Plate Prior to Analysis

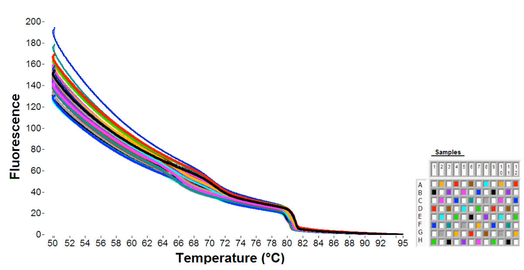

Figure 4: Automatic Detection of the Probe Region

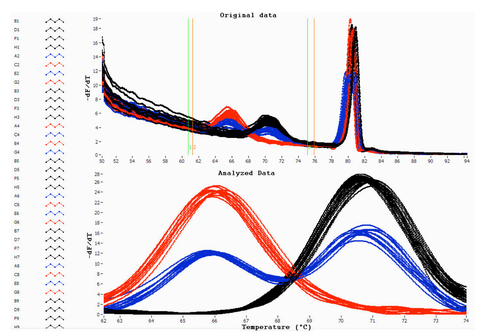

Figure 5: Automatically Genotyped Melting Curves

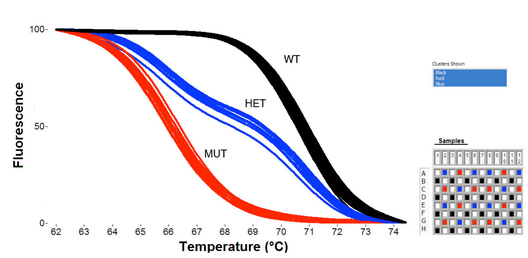

Figure 6: Tms Quantified From Derivative Curves