

**One-Hour Melt-Analysis Genotyping Using Unit-Dose Reagents for
Sample Processing and PCR**

M. Wood, E. Carroll, A. Zuniga, D. Burns, D. Keys
Osmetech Molecular Diagnostics, Pasadena, CA, USA

*Originally Presented at the American Association for Clinical Chemistry
Annual Meeting, San Diego, 2007*



One-Hour Melt-Analysis Genotyping Using Unit-Dose Reagents for Sample Processing and PCR

M. Wood, E. Carroll, A. Zuniga, D. Burns, D. Keys
Osmetech Molecular Diagnostics, Pasadena, CA, USA

*Originally Presented at the American Association for Clinical Chemistry
Annual Meeting, San Diego, 2007*

INTRODUCTION

Most currently available diagnostic kits utilizing PCR methodology have amplification and detection steps designed to take place on board instrumentation for an automated, rapid result. However, these kits utilize several steps before the PCR and detection that are time consuming and increase the opportunity for an error to occur during the assay. The clinical sample must be purified to obtain sufficient target DNA in a buffer matrix free from substances known to interfere with PCR. This sample purification process, particularly with substances such as whole blood, can be time consuming, complex, and require significant hands-on time by the technician. In addition, these kits require the technician to manually assemble the components of the master mix before the addition to a reaction tube, introducing the potential for errors in formulation and increasing the chances of contaminating the assay.

We investigated ways to reduce the complexity of sample purification and master mix preparation in genetic testing using a prototype Factor V Leiden (FV) assay system. The assay was carried out on the Roche LightCycler® within a plastic PCR tube (SensiTube™) possessing physical characteristics compatible with reagent storage, PCR, and fluorescence detection using melting curve analysis. First, we investigated a potential method to minimally-process whole blood samples in the assay by testing fresh whole-blood and archived blood samples in different anticoagulants. We also investigated the use of PCR tubes pre-filled with a subset of PCR reagents so that the only steps required to set up the assay were the addition of two components (sample and FV probe/primer reagent) and capping the tube.

MATERIALS AND METHODS

Sample Processing

Six fresh whole-blood samples (0.5-1 µL) were obtained via finger lancet and diluted 1:30-1:3000 in QuickExtract™ DNA Extraction Solution (Epicentre Biotechnologies). In addition, archived blood samples in heparin, citrate, EDTA, and ACD anticoagulants from 6 individuals were obtained from frozen storage and diluted 1:1000 in QuickExtract™. Each diluted sample was vortexed for 15 seconds, heated at 65 and 98

°C for 1 and 2 minutes, respectively, and cooled to room temperature. The samples were vortexed for 15 seconds after each heating step. Processed samples were assayed immediately.

Pre-Filled Tube Set-up

A mixture of GPRs (PCR buffer, magnesium chloride, dNTPs, KCl, BSA, and *Taq* polymerase) was prepared and 13 µL of this mixture were added to each SensiTube. The filled SensiTubes were stored at 2-8 °C for approximately 24 hours prior to use. Immediately prior to running the assay, 2 µL of the primer /probe mix and 5 µL of the processed sample were added to obtain the final reaction volume of 20 µL.

FV Assay Conditions

The model FV assay is based on fluorescein-based probe chemistry and melt curve analysis utilizing HyBeacon® probes (licensed from LGC, Ltd., UK). LightCycler PCR reactions (20 µL) contained primers (0.5 µM), probe (0.15 µM) and DNA sample in 10 mM Tris buffer (pH 8.3), 50 mM KCl, 3 mM MgCl₂, 1 mM dNTPs, 5 ng/µL BSA. PCR consisted of an initial denaturation step (95 °C, 60 sec) followed by 40 cycles of amplification (55 °C, 10 sec; 72 °C, 10 sec; 95 °C, 5 sec). The samples were cooled to 35 °C for 30 sec and then melting curve analysis was conducted (35-70 °C) to monitor the decrease in fluorescence emission from the probe that occurred upon denaturation of the probe-amplicon complex.

RESULTS

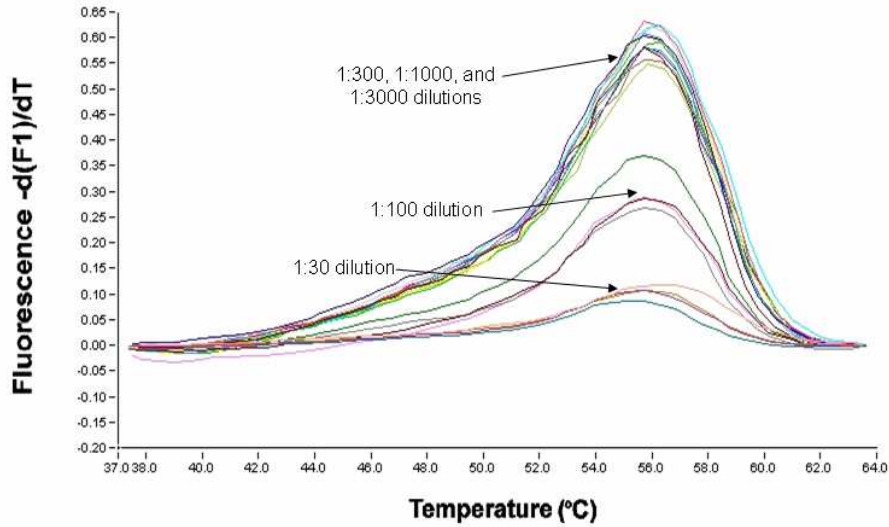
Dilution of Blood in QuickExtract DNA Extraction Solution

A series of dilutions (1:30 to 1:3000) from a single blood sample were made in QuickExtract and processed according to the product protocol. The processed sample dilutions were tested (4 replicates at each dilution) in the FV PCR assay. T_m results were consistent across the dilution range at ~55.5 °C (wild-type FV genotype) with CV's below 1% (Table 1). Peak areas were lowest at the 1:30 dilution level and increased with each dilution factor until 1:300, at which point the peak areas leveled off (Table 1 and Figure 1).

Table 1: Factor V Assay Results Obtained From Serially Diluted Blood Processed in QuickExtract Buffer

Dilution	T _m (°C) Results		Peak Area Results	
	Mean	CV, %	Mean	CV, %
30	55.5	0.7	0.7	19.5
100	55.5	0.1	1.9	16.0
300	55.5	0.1	3.9	5.0
1000	55.7	0.1	4.1	4.6
3000	55.7	0.2	3.9	4.6

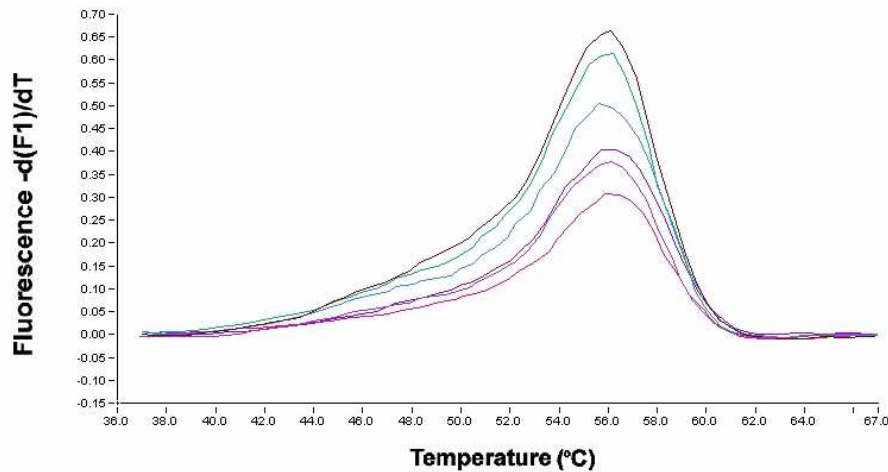
Figure 1: Melt Curves Obtained From Dilution of Blood Samples in QuickExtract Buffer



Evaluation of Fresh and Archived Blood Samples using QuickExtract Method

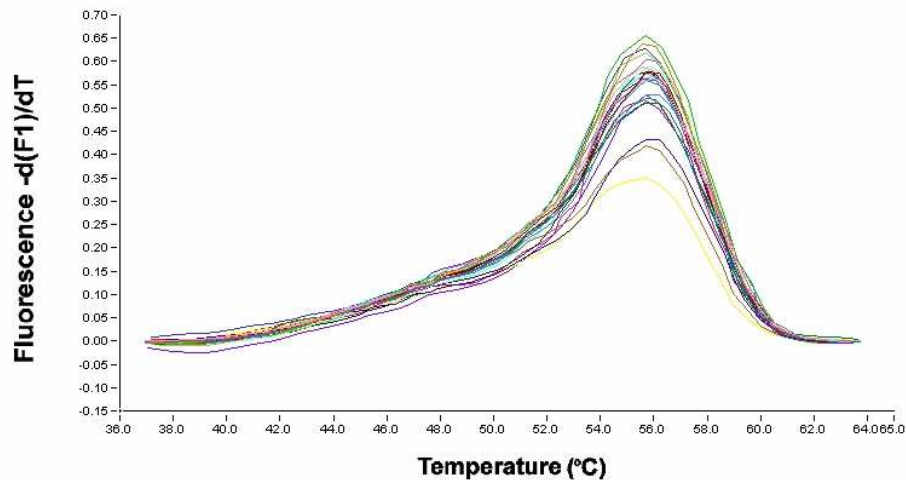
Six fresh whole blood samples were processed in the QuickExtract buffer at a 1:1000 dilution and tested in the FV assay. All six samples generated melt curves consistent with initial testing, with average $T_m = 55.7$ °C and peak areas averaging 2.9 (Figure 2).

Figure 2: Melt Curves Obtained From 1:1000 Dilution of Six Different Lancet Blood Samples in QuickExtract Buffer



Six individual archived blood samples collected in sodium citrate, sodium heparin, K₂EDTA, and acid citrate dextrose (ACD) were also processed in the QuickExtract buffer at a 1:1000 dilution and tested in the FV assay. During sample preparation it was noted that two of the ACD blood samples (donors 4 and 5) were very pale in color, indicating that they had particularly low concentrations of blood cells. These two samples were not included in the analysis. The remaining 22 samples were evaluated and generated melt curves with T_ms and peak areas averaging 55.4 °C and 3.4, respectively (Figure 3).

Figure 3: Melt Curves Obtained From 1:1000 Dilution of Six Different Archived Blood Samples with 4 Different Anticoagulants in QuickExtract Buffer



Evaluation of Fresh and Archived Blood Samples with Pre-Filled SensiTubes

The same set of archived blood samples were processed using the method described above and tested in SensiTubes that had been filled with a GPR mixture. Results are summarized in Table 2. All 24 samples tested were positive for wild-type FV genotype (T_m range = 55.3 – 56.7 °C), a 100% concordance with prior testing using purified DNA from these blood samples. Four of the samples generated melt peaks that were not detected by the LightCycler software; manual T_m analysis was used to determine T_m's of these samples.

Table 2: FV Assay Results from QuickExtract Blood Samples Tested in Pre-Filled SensiTubes

Results by Donor

Donor	T _m (°C) Results		Peak Area Results	
	Mean	CV, %	Mean	CV, %
1	55.8	0.7	1.0	37.9
2	55.9	0.9	1.1	35.4
3	56.2	0.8	1.0	42.2
4	55.8	0.8	1.6	26.2
5	55.6	0.4	1.0	56.3
6	55.7	0.7	1.4	45.9

Results by Anti-Coagulant

Anti-Coagulant	T _m (°C) Results		Peak Area Results	
	Mean	CV, %	Mean	CV, %
Sodium Citrate	55.9	0.7	0.9	56.5
Heparin	55.8	0.5	1.4	28.2
EDTA	55.8	0.8	1.3	30.5
ACD	55.8	1.0	1.1	56.2

CONCLUSIONS

This study has shown that a simple, quick, and inexpensive blood sample preparation method (QuickExtract DNA Extraction Protocol) can be coupled successfully to a PCR-based genotyping assay. The total time required to obtain the sample and prepare it for PCR was less than 5 minutes and the total time required from blood sample collection to assay result was less than 1 hour. This compares favorably with assays that use purified DNA samples since typical DNA extraction protocols alone require approximately 1 hour to complete. In addition, no interference was observed with this method from blood stored in different anti-coagulants, including heparin (a known PCR inhibitor).

Based on the average white blood cell concentration in whole blood ($5 \times 10^6/\text{mL}$), the input of cells per assay was ~25 cells (50 FV gene copies). This high sensitivity allowed for the use of very small volumes of blood and alternative sample collection methods such as the finger lance. This method is less stressful for a patient (compared to standard phlebotomy) and is also quicker and inexpensive.

The FV assay performed impressively when coupled to the QuickExtract protocol. All samples (archived and lancet) gave unambiguous positive results for wild-type FV genotype. T_m's with the QuickExtract method were within the expected range for the wild-type FV genotype and showed impressive precision.

Finally, the QuickExtract method was successfully used in conjunction with a new assay configuration with pre-filled SensiTubes. The entire procedure requires only three pipetting steps per sample, specifically placement of blood in the sample preparation reagent, sample delivery to the pre-filled PCR tube, and addition of primer/probe reagent. All of the samples evaluated gave unambiguous positive results for wild-type FV genotype.

For Research Use Only. Not for Use in Diagnostic Procedures.