Improved speed and accuracy of medical assessment tools combined with reduced cost and easy operation are important factors in Point-of-Care testing. In the future such tests should be carried out directly at the physician's office or even at home by patients monitoring their health conditions. Therefore portable test systems working directly from crude samples will render obsolete the need for sending specimen to clinical laboratories. Enabling the physician to assess a medical condition on-site will greatly reduce pressures time in patient treatment, and lead to significant saving in health care cost. As an example when prescribing Warfarin at first, the metabolic compatibility of the patient should be assessed to avoid risk of adverse side effects. Here we report a fully isothermal on-site genetic test system for Warfarin dosing, which works directly from crude blood samples without the necessity of time-consuming sample purification steps. Using an easy to use ESE-Quant TS reader fully interpreted results are obtained within less than 60 minutes by simply pushing one button. The underlying DNA based SmartAmp 2 nucleic acid detection technology is highly specific and its affordability makes it an ideal candidate to be deployed in every physician's office and research laboratories.

"Although still a prototype assay, we are working on improved lysis condition for our method to convert the assay into a true one-step reaction using also other starting materials than blood."

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Rapid Genetic Testing for Warfarin Dosing on an ESE-Quant TS Reader
An Example for Future Point-of-Care Solutions to Personalised Medicine

INTRODUCTION

Warfarin, also known under its brand names Coumadin, is one of the most important anticoagulants presently in use to stop blood clotting in medical treatment for thrombotic disorders. In the USA only, it has been estimated that about 2 million people start Warfarin treatment every year, out of which however some 85,000 patients suffer from bleeding and additional 17,000 from strokes as serious side effects caused by inappropriate initial dosing of the drug. Recently clinical data from various studies have supported the idea that genetic testing prior to Warfarin dosing could substantially reduce the risk of bleeding and clotting events.

Such genetic tests determine specific somatic mutations (Single Nucleotide Polymorphisms or SNPs) in the VKORC1 (vitamin K epoxide reductase) and CYP2C9 (cytochrome P450 2C9) genes. The VKORC1 gene encodes the enzyme on which Warfarin exerts its effect, and a SNP in the promoter of that gene (VKORC1 – 1639 G/A) allows it to identify patients more sensitive to Warfarin, and hence requiring a lower dose than average patients. The gene product of the CYP2C9 gene is primarily responsible for metabolizing Warfarin in the liver. Two SNPs in the CYP2C9 gene (CYP2C9*2 (C/T) and CYP2C9*3 (A/C)) have been shown to slow down Warfarin metabolism, and thus leading to an accumulation of the drug in the blood over an extended time. Therefore patients with a slower Warfarin metabolism typically require a lower dose of Warfarin. Although throughout dose adjustment regimens for Warfarin based on genetic testing still have to be developed, the American Food and Drug Administration (FDA) highlighted already in 2007 that 'the opportunity for healthcare providers to use genetic tests to improve their initial estimate of what is a reasonable Warfarin dose for individual patients' [1], making Warfarin a prime target for genetic testing. Following the FDA initiative we and others are working on easy to use rapid genetic tests to be performed right at the physician's office. We focused on a fully isothermal process that allows detection reactions directly from blood in combination with a dedicated reader yielding final results within 60 minutes from taking the blood sample.

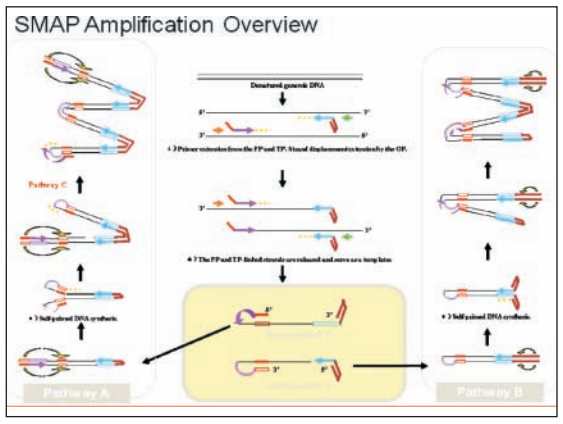

Figure 1: SmartAmp 2 reaction. For details refer to text.

THE TEST SYSTEM

Mitani et al. at the RIKEN Omics Centre in Japan developed the isothermal SmartAmp 2 genotypic method [2] that is characterised by an asymmetric primer design for effective background suppression. The reaction mechanism is outlined in *Figure 1*: An inner primer set comprising a 'Folding' or FP primer and a 'Turning' or TP primer initiates the reaction by hybridizing to the opposite strands of a target region. Linear primer extension products from the FP and TP primers are then released from their templates in a second primer extension reaction driven by a set of outer primers that hybridize downstream of the FP and TP primers and the Aac DNA polymerase with its high strand displacement activity. Due to the special features of the FP and TP primers, single-stranded primer extension products from those primers will refold at their 3' ends to form new priming sites (Intermediate Products 1 and 2). Depending on their structures at the 3' end Intermediate Products 1 and 2 will maintain their own amplification in a cautious process driven by the high strand displacement activity of Aac DNA polymerase (Reaction Pathway A or B). The amplification process can easily be monitored by the incorporation of fluorescent dyes. Using fluorescence labelled primers (so-

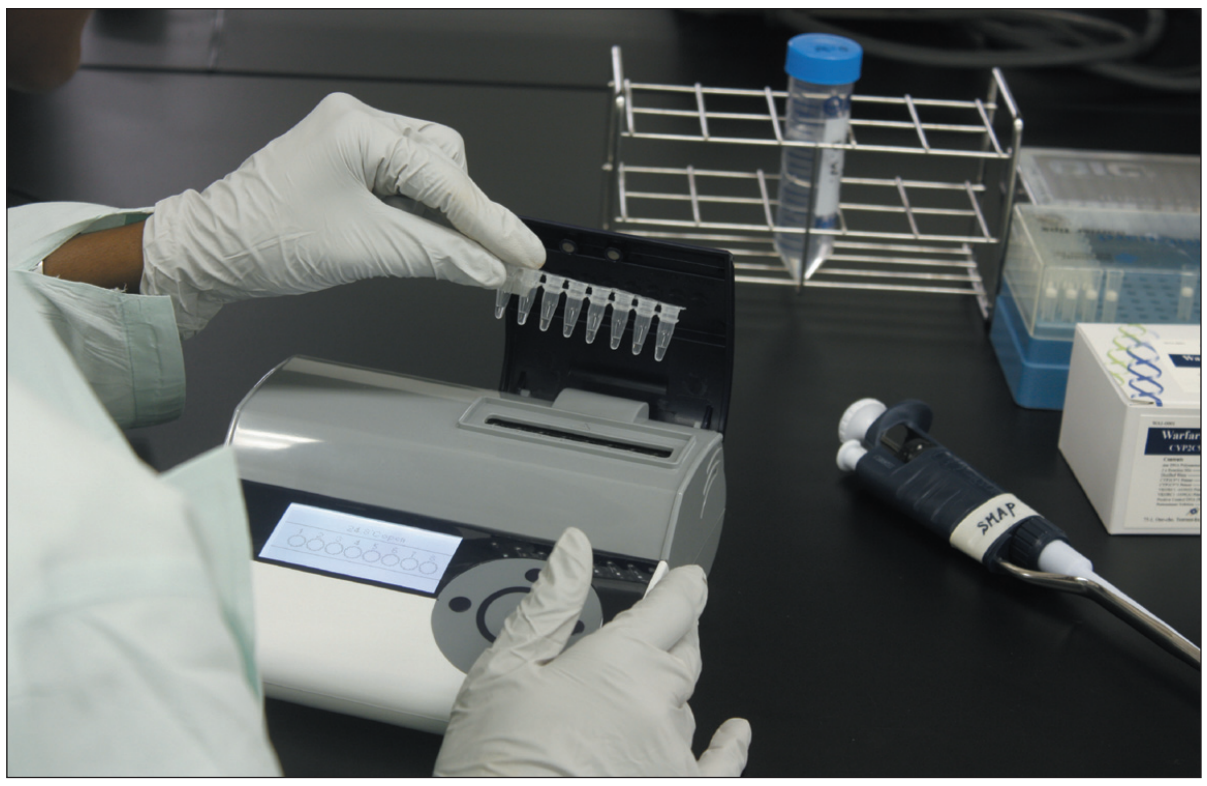

Figure 2: ESE-Quant TS is a portable fluorescence tube scanner for real-time DNA analysis, it has about half the footprint size of a computer laptop.

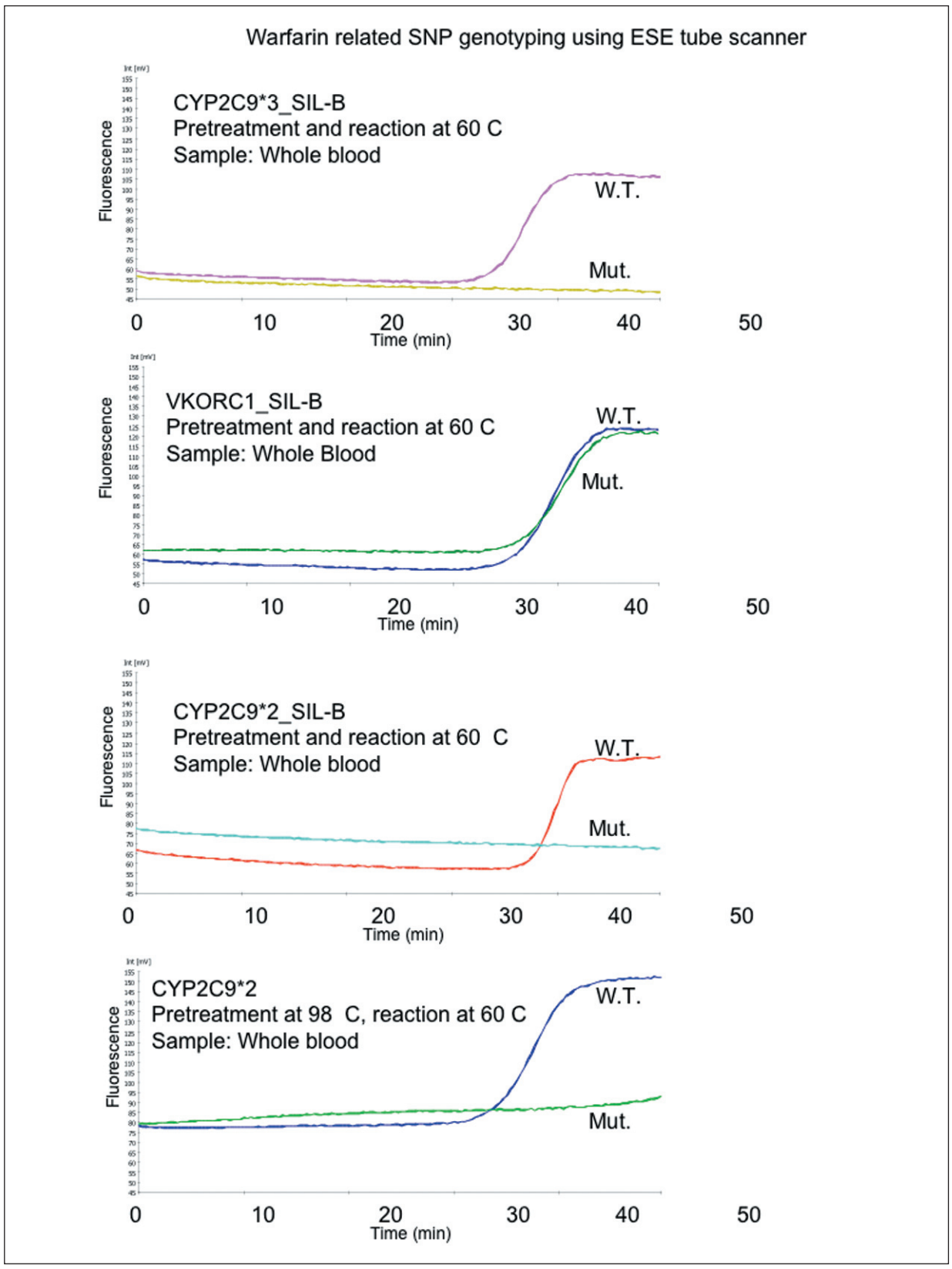

*Figure 3: Top to bottom: 3A, 3B, 3C, 3D. SNP detection reactions conducted in the ESE-Quant TS reader at 60°C for up to 50 minutes monitoring for wild-type and SNP specific dyes in real-time (3A-3C). Figure 3D: Control experiment for CYP2C9*2 including the alkali pretreatment step at 98°C prior to amplification at 60°C in the ESE-Quant TS reader.*

called Exciton Primers) as 'sequence specific dyes' as the discriminator of wild type and mutated DNA, SmartAmp 2 allows for rapid and reliable genotyping in two-colour single tube reactions. With it's throughout isothermal process SmartAmp 2 is an optimal application for the new ESE-Quant TS reader.

The ESE-Quant TS reader and diagnostic prototype test system *(Figure 2)* is a portable and highly sensitive real-time fluorescence detection system. Fully interpreted data (as well as raw data) can be obtained by the operator pressing a single button. The miniaturised reader allows for testing of up to eight samples in parallel and simultaneous readout of two dyes at each position. Its sensitivity translates into rapid time-to-result experiments. The reader is approximately 20cm x 15cm x 8cm in size and just about 0.6kg in weight. ESE's miniaturised fluorescence sensor technology used in the ESE-Quant TS reader is based on LEDs that have been described before [3, 4]. Optionally the reader can be fully battery operated and be used as a stand alone analytical instrument due to its internal memory, display and keypad as for example needed for use in ambulances or field tests. For laboratory use the instrument can, however, also be connected to a power supply and a computer by a standard USB cable for extended data analysis and storage.

RESULTS AND DISCUSSION

Aomori et al. have published specific SmartAmp 2 primer sets to detect SNPs in CYP2C9*2, CYP2C9*3, and VKORC1-1639 [5] that were used to develop our rapid genotyping test for Warfarin dosing in combination with the ESE-Quant TS reader and diagnostic prototype test system. Since the ESE-Quant TS reader is designed for isothermal reactions only, we revised the SmartAmp 2 assays from Aomori et al. by use of the SmartAmp Isothermal Lysis Buffer (SIL-B) for direct cell lysis [6]. To perform the test, 2 µl of blood obtained with the informed consent of the donors are mixed with 4µl of SIL-B buffer and incubated at 60°C for 3 minutes in the ESE-Quant TS reader.

After cell lysis 2 µl each of the lysis reaction are mixed with 28 µl of SNP-specific reaction mixtures containing the Aac DNA polymerase, specific primer sets, dNTPs, and reaction buffer. The Warfarin dosing assay requires three reactions to monitor each of the SNPs in CYP2C9*2, CYP2C9*3, and VKORC1-1639 separate reactions. The SNP detection reactions (10 µl reaction volume for the examples shown in the figures) are again conducted in the ESE-Quant TS reader at 60°C for up to 50 minutes monitoring wild type and SNP specific dyes in real-time *(Figure 3A to C)*. Clear amplification signals against minimal background amplification indicate the genotype of the patient, where amplification equals direct detection in SmartAmp 2 assays. While traditional SmartAmp 2 assays require an alkali pretreatment step at 98°C followed by amplification at 60°C, our modified assays using SIL-B buffer confers the advantage of a single temperature assay greatly reducing probe handling and instrumentation. A control experiment for CYP2C9*2 including the alkali pretreatment step at 98°C prior to amplification at 60°C in the ESE-Quant TS reader, showed that our new fully isothermal test has the same sensitivity and reaction speed as the conventional method *(Figure 3D)*.

SUMMARY AND FUTURE OUTLOOK

The ESE-Quant TS reader has a capacity of 8 reactions at the same time. Hence the instrument could be used to test two blood samples for Warfarin dosing at the same time including positive and negative reaction controls. With more dedicated SmartAmp 2 assays becoming available in the future, the assay concept described here could be extended to the testing other SNPs important for personalised medicine. With its entirely isothermal process, our assay system is a great step forward over other established assays such as the PCR that require far more complex instrumentation and prolonged hands-on bench time. Although still a prototype assay, we are working on improved lysis condition for our method to convert the assay into a true one-step reaction using also other starting materials than blood. Convenient, easy to carryout, assays in combination with an affordable device such as the ESE-Quant TS reader promise optimal solutions for researchers' standard assays, medical doctors' daily routines in patients care, and outdoor testing by field scientists.

NOTICE

The Warfarin dosing assay described in here is not approved by any authority as a medical device or otherwise as a diagnostic test.

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