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Integrated amplification microarray system in a lateral flow cell for warfarin genotyping from saliva

Thomas Sebastian [a,*], Christopher G. Cooney [a], Jennifer Parker [a], Peter Qu [a], Alexander Perov [a], Julia B. Golova [a], Lindsay Pozza [b], Rafal M. Iwasiow [b], Rebecca Holmberg [a]

[a] Akonni Biosystems, Inc., 400 Sagner Avenue, Suite 300, Frederick, MD 21701, United States
[b] DNA Genotek, Inc., 2 Beaverbrook Road, Kanata, Ontario K2K 1L1, Canada

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ABSTRACT

Background: Genetic polymorphisms in the CYP2C9 and VKORC1 genes have been linked to sensitivity of the anticoagulant drug warfarin. The aim of this study is to demonstrate a method for warfarin sensitivity genotyping using gel element microarray technology in a simplified workflow from sample collection to analysis and detection.

Methods: We developed an integrated amplification microarray system combining PCR amplification, target labeling, and microarray hybridization within a single, closed-amplicon "lateral flow cell" for genotyping three single nucleotide polymorphisms (SNPs) that influence warfarin response. We combined nucleic acid extraction of saliva using the TruTip technology together with the lateral flow cell assay and with fully automated array detection and analysis.

Results: The analytical performance of the assay was tested using 20 genotyped human genomic DNA samples and found to be sensitive down to 330 input genomic copies (1 ng). A follow-up pre-clinical evaluation was performed with 65 blinded saliva samples and the genotyping results were in agreement with those determined by bidirectional sequencing.

Conclusions: Combined with the use of non-invasive saliva samples, rapid DNA extraction, the lateral flow cell, automatic imaging and data analysis provides a simple and fast sample-to-answer microarray test for warfarin sensitivity genotyping.

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1. Introduction

Warfarin is widely used as an anti-coagulant for the treatment and prevention of arterial and venous thromboembolism [1,2]. However, the drug is associated with the greatest number of serious adverse drug events over the past two decades, due to its narrow therapeutic index and the substantial inter-individual variability in dosing requirements [3,4]. There is increasing evidence indicating that a number of factors affect warfarin dosing, including non-genetic and genetic factors [2,4–6].

Genetic factors affecting warfarin pharmacokinetics and pharmacodynamics account for roughly 40% of warfarin dosing variability and stem primarily from two genes: warfarin's target gene, *VKORC1* [7,8] and the gene of its main metabolizing enzyme, *CYP2C9* [9]. Identifying individuals with the polymorphisms that produce various responses in warfarin therapy and the concomitant adjustment of their warfarin dose is expected to confer substantial benefit to patients and in cost savings to the healthcare system. Clinical studies have shown a correlation between steady-state warfarin dose and allelic variants of *CYP2C9* and *VKORC1* [4,10]. To encourage the use of pharmacogenetic testing for patients starting warfarin therapy, the US Food and Drug Administration (FDA) mandated the inclusion of a warning label on warfarin packaging regarding the relationship of safe and effective dosage to individual patients with mutations in these specific genetic regions [11,12].

The anticipated need for warfarin genotyping prompted the development of numerous laboratory-developed clinical assays and commercial platforms focused on CYP2C9 430C > T (CYP2C9*2), CYP2C9 1075A > C (CYP2C9*3) and VKORC1 -1639 (or 3673) G > A genotyping [13–15]. Comparative studies of the several warfarin genotyping commercial platforms reported that the assays were found to differ in turnaround time and hands-on time, requirements for amount of input genomic DNA, accuracy and cost, specialized equipment usage and other factors that might makes each assay more favorable in different settings [13,14,16,17]. A wider adoption of the warfarin assay in clinical laboratories requires the development of testing platforms that can

Abbreviations: CYP2C9, cytochrome P-450 variant 2C9; VKORC1, vitamin K epoxide reductase complex-1; SNP, single-nucleotide polymorphism; PCR, polymerase chain reaction.

* Corresponding author. Tel.: +1 301 698 0101; fax: +1 301 698 0202.
E-mail address: tsebastian@akonni.com (T. Sebastian).

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reduce the cost of reagents and consumables, expensive supporting instrumentation, and the amount of handling required. Recent publications reported on the development of a few warfarin genotyping platforms offering a lower cost solution, but require multiple processing steps and create the potential for amplicon cross contamination [18,19].

DNA microarrays are a powerful genomic tool that can be applied to biomedical and clinical research [20,21]; however, a simplified workflow is required for a wider adoption of the microarray platform in diagnostic applications. Typical protocols incorporating microarray detection generally involve several steps, including nucleic acid purification, PCR amplification, amplified target purification and/or modification (e.g. fragmentation, labeling and/or single-stranded target generation), target hybridization, array washing, drying, imaging and complex manual data analysis. Chandler et al. reviewed the recent methods for condensing this workflow by combining target amplification, labeling, and microarray hybridization into a single, closed-amplicon reaction chamber, and provided examples of this approach in infectious disease diagnostics [22]. A key element of this approach is the three dimensional gel element microarrays which enable an increase in hybridization efficiency and detection sensitivity compared to planar arrays [23,24] and is well suited to a lower cost imaging system. We recently reported on a valve-less lateral flow cell with a simple consumable architecture that supports target amplification and microarray hybridization in the same chamber [25]. This lateral flow cell also incorporates post hybridization wash steps while retaining an entirely closed-amplicon system, thus minimizing the potential for sample or amplicon cross-contamination. These advancements simplify the microarray workflow without custom instrumentation and increase its applicability for diagnostic applications.

The objectives of this study were therefore to demonstrate the application of the integrated amplification microarray in a lateral flow cell for genotyping the *2 or *3 alleles of CYP2C9 and the VKORC1 allele to determine warfarin sensitivity, with an emphasis on clinical ease-of-use and reduced complexity. In order to develop a simplified workflow for routine warfarin genotyping, we combined the target amplification, labeling, and microarray hybridization within a single, closed-amplicon microfluidic chamber with fully automated array imaging and analysis. The assay performance of this system was optimized and evaluated using a series of genomic DNA (gDNA) samples. The final workflow incorporated saliva samples extracted using the rapid DNA extraction capability of the TruTip technology for an end-to-end solution. This approach was finally applied to the genotyping of a set of 65 blinded saliva samples for pre-clinical evaluation.

2. Materials & methods

2.1. Source DNA

2.1.1. Purified gDNA samples

A set of 20 genotyped human genomic DNA samples (NA17075, NA17119, NA17204, NA17207, NA17210, NA17214, NA17215, NA17216, NA17219, NA17220, NA17221, NA17222, NA17229, NA17243, NA17247, NA17252, NA17254, NA17259, NA17285, NA17290) were obtained from the Coriell Institute Cell Repository (Camden, NJ). Each purified DNA sample is either wild type (WT), mutant (MUT), or heterozygous (HET) for each SNP target, CYP2C9*2, CYP2C9*3 and VKORC1. DNA concentration was estimated on a NanoDrop 1000 (Thermo Scientific, Wilmington, DE) before use.

2.2. Saliva samples

Sixty-five de-identified saliva samples collected and preserved using the Oragene®•Dx self-collection kit were obtained from DNA Genotek, Inc. (Ontario, Canada). The warfarin genotype information was blinded in these samples. DNA was extracted and purified from the saliva samples using Akonni's TruTip gDNA Saliva Kit (Cat No. 300-20431) according to manufacturer's recommendations. Purified DNA was eluted from the TruTip matrix with 100 μl of elution buffer. The quantity and quality of the DNA were determined using a NanoDrop 1000 and quantification was verified by qPCR using the Quantifiler® Human DNA Quantification Kit (Life Technologies, Carlsbad, CA). DNA was stored at −20 °C until use.

2.3. Microarray design and manufacture

Microarray probes were designed for each SNP target, two hybridization probes (one probe per allele) (Table 1). Probe sequences with artificial mismatch positioned relative to the SNP target were included for enhanced discrimination of single nucleotide polymorphisms by DNA hybridization [26]. All probes for microarray manufacturing were synthesized by Akonni with a custom 3′-methacryamido-linker and purified to >90% purity by HPLC. Control probes include Cy3-labeled 8-mer oligonucleotides for positional reference beacons of the array and manufacturing quality control and a plant sequence with no known homology to any human DNA sequence as a negative control probe. These probes provided an internal control of reagents, array integrity, and non-specific hybridization. Microarrays were manufactured

Table 1
DNA sequence of all PCR primers and microarray probes used in the assay.

Primer ID	Sequence (5′–3′ direction)	Tm (°C)	Amplicon size, nt
CYP2C9*2-F	[Cy3] ATGGAAGGAGATCCGGCGTTT	62.6	155
CYP2C9*2-R	AGGTCAGTGATATGGAGTAGGG	62.7	
CYP2C9*3-F	CCAGGAAGAGATTGAACGTGTG	62.7	187
CYP2C9*3-R	[Cy3} ACTTACCTTGGGAATGAGATA	56.7	
VKORC1-F	TGG GAA GTC AAG CAA GAG AAG ACC	64.6	75
VKORC1-R	[Cy3]TGCTAGGATTATAGGCGTGAGCCA	64.6	
Probe ID	**Sequence (5′–3′ direction)**	**Tm (°C)**	**Probe Length**
CYP2C9*2-3L	TCTTGAACAC**G**GTCCTCAATG	59.5	21
CYP2C9*2-4L	TCTTGAACAC**A**GTCCTCAATGC	60.1	22
CYP2C9*3-5L	TCCAGAGATAC**A**TTGACCTTCTC	60.9	23
CYP2C9*3-6L	TCCAGAGATAC**C**TTGACCTTCT	60.1	22
VKORC1-7S_A	TTGaCC**G**GGTGCG	44	13
VKORC1-8S_A	ATTGaCC**A**GGTGCG	43.7	14
Negative control probe	TCT TCT TCC TCC TCC TCG TC	60.5	20

Bold letters in the probe sequences indicate SNP and lowercase letters in the probe sequences indicate intentional mismatches versus the genomic template. The melting temperature (Tm) was calculated with the Oligo Calc.

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