

## A Methodologic Comparison of Invader and Autogenomics INFINITI in Factor-V Leiden and Prothrombin Gene Mutation Testing

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### Abstract

**Background:** Collectively, factor V Leiden (FVL, rs6025) and prothrombin gene mutation (PGM, rs1799963) are responsible for the majority of inherited thrombophilic states in Caucasians. Patients with inherited thrombophilias are at a greater risk of unprovoked venous thromboembolic events. As such, testing for these abnormalities has become standard of care in the appropriate clinical context.

In this study, we compare the workflow and performance of a non-PCR based method, the Invader<sup>®</sup> assay, and the INFINITI<sup>®</sup> assay in FVL and PGM testing. The driving factor behind the methodological comparison was the need to gain efficiency in our molecular diagnostic pipeline. If viable, the INFINITI<sup>®</sup> platform would allow for rapid expansion of the menu of molecular diagnostic tests performed by our institution.

**Methods:** To establish concordance between the two assays, forty four and thirty one specimens that had been previously tested with the Invader<sup>®</sup> assay for FVL and PGM, respectively, were identified. These specimens were subsequently tested on the INFINITI<sup>®</sup> platform, and results from the two assays were compared.

**Results:** The INFINITI<sup>®</sup> Assay produced 100% concordance with the Invader<sup>®</sup> assay in both FVL (44/44) and PGM (31/31) testing.

**Conclusion:** These results indicate that the INFINITI<sup>®</sup> platforms can reliably genotype FVL and PGM.

**Keywords:** Gene mutation testing; Fluorescein; Thrombophilia

### Introduction

The factor V Leiden (FVL, rs6025) and the prothrombin gene mutation (PGM, rs1799963) are responsible for the majority of inherited thrombophilic states in Caucasians [1-4]. FVL and PGM mutations are single nucleotide base changes from guanine to adenine, which occur at position 1691 (G1691A) and at position 20210 (G20210A), respectively [5,6]. The result of these mutations promotes a hypercoagulable state, predisposing affected individuals to venous thromboembolism [7]. The FVL mutation causes an amino acid substitution of arginine for glutamine in the gene product, which alters the ability of activated protein C to cleave activated factor V and VIII, resulting in a prothrombotic state. Similarly, the PGM leads to increased serum levels of prothrombin, the penultimate product of the coagulation cascade [2]. The risk of venous thromboembolism is dependent on zygosity and may result in recurrent thrombotic events, especially considering that many affected individuals are young [5]. Therefore, genetic testing is necessary to determine the presence of FVL or PGM and establish zygosity [4-8]. Genotyping also helps guide clinical therapeutic decisions and provides critical information for other family members who may be at risk of harboring these mutations [9].

PCR based methods have conventionally been used for FVL and PGM testing, however a variety of alternative assays have also been developed. These include the non-PCR based Invader<sup>®</sup> assay, which has been used for a variety of molecular tests including FVL and PGM. The Invader<sup>®</sup> assay relies on Cleavase enzymes; it provides linear signal amplification genomic DNA and does not require prior PCR amplification of the target [10].

In contrast, the INFINITI<sup>®</sup> System Assay for Factor II-V Leiden is a microarray based method. The initial step in both assays is DNA extraction from whole blood specimens. However, subsequent steps of the INFINITI<sup>®</sup> assay include multiplex PCR followed by analyte specific

primer extension and hybridization and analysis of a microarray. The strength in this platform is the large size of the available testing menu sharing similar analytic pathways, thus decreasing the need for cross-training and separate pipelines. This study evaluates the concordance of genotyping results between the Invader<sup>®</sup> and INFINITI<sup>®</sup> assays.

### Materials and Methods

#### Sample preparation

Specimens included in this study consisted of those obtained from patients who presented with thrombosis and patients at risk of a thrombotic event based on clinical or environmental factors or family history. Whole blood specimens were collected in EDTA vacutainer tubes. A total of seventy five specimens were analyzed, forty four of which were tested for FVL, and thirty one were tested for PGM. Replicates of these samples were tested on different days and with different reagent lots.

Genomic DNA was extracted from whole blood using the Qiagen Bio Robot EZ1/Geno-M6 kit (QIAGEN Valencia, CA) according to manufacturer instructions. The extracted DNA was quantified with the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The minimum required DNA concentration was 10

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ng/uL, and the minimum required absorbance ratio at 260 nm/280 nm was 1.7-1.9.

### Invader® assay

For analysis with the Invader® assay (Hologic, Bedford, MA formerly Third Wave Technologies, Inc. Madison, WI), five microliters of a reaction mix (PEG 8000, MOPS pH 7.5, ProClin 300, and 0.1umol/L Invader oligonucleotide) were added to each well of a 96 well microtiter plate. Ten microliters of extracted DNA were then added to each well. Each run included wild type, heterozygous, homozygous, and molecular grade water controls. The heterozygous and mutant controls consisted of synthetic oligonucleotides. 20 uL of Chill-Out 14 (MJ Research, Watertown, MA) was then overlaid in each mixture. No target blanks were used to determine probe-specific background values. He specimens were then denatured in a Techne TC-512 Thermal Cycler (Techne, Burlington, NJ) at 95°C and subsequently cooled to 63°C. At this step, 5 uL of Cleavase enzyme was added to each reaction. The microtiter plate was then incubated for four hours at 63°C. The microtiter plate was then analyzed with a Tecan GENios fluorescence reader (Tecan, Männedorf, Switzerland). Fluorescein and Redmond RedTM were utilized as fluorescent dyes with excitation/emission spectra at 485/535 nm and 560/612 nm, respectively. Fluorescein was the indicator of mutant target, while the Redmond RedTM was the fluorochrome indicator of wild type sequence.

### INFINITI® assay

The INFINITI® System Assay for Factor II-V Leiden (Autogenomics, Inc., Vista, C).

- a) Detects and genotypes factor II (prothrombin) G20210A and factor V Leiden G1691A point mutations. This assay involves several steps: a) DNA extraction from human blood samples,
- b) Multiplex PCR amplification,
- c) Analyte specific primer extension with incorporation of fluorescent labels,
- d) Hybridization to a microarray,
- e) Signal analysis [11].

For analysis with the INFINITI® assay, the minimum required DNA concentration was 25 ng/uL, and the minimum required absorbance ratio at 260 nm/280 nm was 1.7-2.0. A PCR master mix was prepared according to the instructions for use, of which 18 uL were dispensed to each well of a 24-well microtiter plate. 2 uL of prepared extracted DNA was then added to each well. A spin amplification reaction was conducted in a Heraeus™ Multifuge™ X1 (Thermo Fisher Scientific, Wilmington, DE) followed by multiplex amplification in a Techne Flexigene thermocycler (Techne, Burlington, NJ). The plate was first heated to 95°C for 4 minutes and subsequently cooled to 56°C for 15 seconds. It was then heated to 72°C for 15 seconds, and a total of forty cycles were performed. After completion of these cycles, the plate was held at 4°C. The following automated steps were then performed by the INFINITI® Analyzer. Fluorescent labels were incorporated into the targets via analyte specific primer extension. The targets were hybridized to the microarray, which was washed. Signal detection and analysis was conducted by the INFINITI® software. Results were reported as an allele variant, and the threshold ratio of mutant to background DNA was 0.1.

### Results

A total of 75 samples (44 FVL and 31 PGM) derived from in-house

specimens were analyzed. The results are summarized in Tables 1-3. 100% concordance between the INFINITI® and Invader® assays was obtained. These results demonstrate that the INFINITI® assay can reliably detect and genotype FVL and PGM.

### Discussion

In the early 1990s, the diagnosis of an inherited thrombophilia was detected in only a small cohort of patients, 5-15%, with venous thrombosis [7]. These inherited thrombophilias consisted of defects in antithrombin, protein C, and protein S. Since then, additional causes of inherited thrombophilias have been identified, notably the factor V Leiden (FVL) and prothrombin mutations.

FVL is caused by a single nucleotide base changes from guanine to adenine at position 1691 (G1691A) and has been identified in 40-60% of patients with inherited thrombophilia [1-3]. These patients historically demonstrate a decreased response to the anticoagulant activated protein C (APC) with an autosomal dominant phenotype, for which FVL is also known as APC resistance. APC is a plasma serine protease which acts in concert with free protein S to cleave active forms of coagulation factors V and VIII (FVa, FVIIIa). FVL decreases the affinity of the binding site for APC/S complex thereby decreasing proteolysis of the activated factors and producing a prothrombotic state [5]. The single point mutation responsible for this APC resistant phenotype can be heterozygous or homozygous and carries a risk of thrombosis that varies with zygosity. This risk is 80-fold increased in homozygous individuals compared to the 7-fold increase in heterozygous individuals.

The genetic variant of prothombin, which also consists of a single G to A base change, is located at position 20210 and referred to as G20210A [4]. The prothrombin gene mutation, PGM, is found in 18% of patients with familial thrombophilia [6]. In heterozygotes with PGM, prothrombin antigen and activity are increased by about 30%

INFINITI® Assay	Invader® Assay		
	Mutation Detected	Mutation Not Detected	Total
Mutation Detected	20	0	20
Mutation Not Detected	0	24	24
Total	20	24	44

$\kappa = (20+24)/44 = 1$  Chi square ( $\chi^2$ ) = 44 (p < 0.0001)

Table 1: FVL Concordance Results.

INFINITI® Assay	Invader® Assay		
	Mutation Detected	Mutation Not Detected	Total
Mutation Detected	16	0	16
Mutation Not Detected	0	15	15
Total	16	15	31

$\kappa = (16+15)/31 = 1$  Chi square ( $\chi^2$ ) = 31 (p < 0.0001)

Table 2: PGM Concordance Results.

Assay	Genotype	No. Cases
Factor V Leiden	Wild-type	24 (55%)
	Heterozygote	14 (32%)
	Homozygote	6 (13%)
Prothrombin gene	Wild-type	15 (48%)
	Heterozygote	12 (39%)
	Homozygote	4 (13%)

Table 3: Summary of detected genotypes.

compared to the general population, conferring a 2.8-fold increased risk for venous thrombosis.

FVL and PGM genotyping is a common molecular diagnostic test with clinical significance in the work-up of suspected thrombophilia. Genotyping is important to distinguish these mutations from other causes of venous thrombosis, some of which may mimic inherited APC resistance, such as antiphospholipid antibodies [12]. In addition, results of hypercoagulable work-ups help to guide management of patients with venous thrombosis, including the anticoagulant of choice, duration of therapy and role of prophylactic anticoagulation [9].

Traditionally, assays performed to evaluate FVL and PGM include polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and allele-specific PCR [5]. According to recent College of American Pathologist (CAP) survey data, the majority (65%) of laboratories still utilize a PCR-based test when testing for these mutations. However, a large cohort of laboratories, 20% utilizes the increasingly popular non-PCR dependent Invader® assay. This assay relies on target quantitation via linear signal amplification [10,13]. It can reliably detect single nucleotide changes from genomic DNA that are present in FVL and PGM, and prior target amplification is not needed. Other available methods include coagulation tests, sequencing and matrix-assisted laser desorption/ionization mass spectrometry [14].

The Invader® assay takes advantage of the action of a naturally occurring and engineered class of enzymes known as Cleavases [14-16]. These enzymes cleave unpaired regions of DNA which present when the 5' end of an Invader® DNA probe overlaps with the hybridized 3' end of an allele-specific probe by at least one base pair. The overlapping region corresponds to the single nucleotide polymorphism and forms a complex that is recognized by the Cleavase [10]. This enzyme releases the unpaired DNA sequence, or flap, generating fluorescence by allowing separation of the fluorescent probe from the quencher. The cleaved flap then serves as an Invader® probe, and the reaction repeats itself. Generation of signal specifically relies on Fluorescence Resonance Energy Transfer (FRET), which occurs when energy from an excited donor fluorophore is transferred to an acceptor dye. When the acceptor dye and the donor are in close proximity, then the fluorescence is quenched. However in this assay, cleavage allows separation of the donor and acceptor dyes, producing a detectable fluorescent signal.

The INFINITI® System Assay for Factor II-V Leiden provides an alternative mode of genotyping FVL and PGM [16]. The INFINITI® platform is a microarray-based in vitro diagnostic test that involves proprietary reagents and instrumentation including polymerase chain reaction primers, hybridization matrices, a thermal cycler, an imager, and software for the detection and genotyping of FVL and PGM [11]. This assay utilizes DNA extracted from human blood samples. The purified DNA is then amplified via multiplex PCR, and fluorescent labels are incorporated using analyte specific primer extension (ASPE). These primers hybridize to target DNA alleles and contain complementary sequences, zip codes, which bind to the Bio Film™ chip. These zip codes are immobilized at a predetermined position on the microarray, so that mutational status is determined by the position on the microarray that produces signal. Once hybridized to the microarray, the ASPE primers are washed to remove excess unbound probes, scanned and analyzed.

To the authors' knowledge, no previous studies have compared FVL and PGM testing on the Autogenomics INFINITI® analyzer with existing testing platforms. However, other molecular targets including

Technology	Invader®	INFINITI®
Instrument cost (USD)	75,000	250,000
Cost per test (USD)	611	902
Sample preparation time (min.)	20	20
Overall test time (hr)	4	10
Advantages	Low cost	High throughput, multiple analytes on a single chip
Disadvantages	Limited test menu, lower throughput	Cost

**Table 4:** Comparison of platform characteristics.

KRAS mutational status have been studied on the Autogenomics platform. French et al. tested concordance of colorectal tumors for KRAS mutations by Sanger sequencing and the INFINITI® analyzer [17]. Twenty eight of these samples were concordant between the two methodologies. Additionally, warfarin sensitivity genotyping has also been examined on the INFINITI® analyzer. Babic et al. reported excellent accuracy between the INFINITI® and two other commercially available platforms when utilizing warfarin sensitivity genotyping panels in the testing of 100 samples [18].

Our results confirm that the INFINITI® assay can reliably detect and genotype FVL and PGM. It has similar analytic performance to the Invader® assay, as our results were 100% concordant. Furthermore, in contrast to the Invader® system, the INFINITI® assay is not limited by the quantity of target DNA, as it involves target amplification. The INFINITI® platform provides a high-throughput method capable of detecting multiple targets within one reaction due to its use of multiplex PCR, and this platform allows increased efficiency by the ability to add additional assays with similar workflows. For example, INFINITI® assays for greater than 40 analytes/panels are available many of which are compatible to be run simultaneously. Despite increased initial investment in equipment, the INFINITI® system is a cost-effective platform in high volume institutions (Table 4). In this manner, the INFINITI® technology can expand the menu of molecular diagnostic tests performed by our institution without increased need for additional technical training, space, or resources.

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