Detection of Genomic Polymorphisms Associated with Venous Thrombosis Using the Invader Biplex Assay

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A multi-site study to assess the accuracy and performance of the biplex Invader assay for genotyping five polymorphisms implicated in venous thrombosis was carried out in seven laboratories. Genotyping results obtained using the Invader biplex assay were compared to those obtained from a reference method, either allele-specific polymerase chain reaction (AS-PCR), restriction fragment length polymorphism (PCR-RFLP) or PCR-mass spectrometry. Results were compared for five loci associated with venous thrombosis: Factor V Leiden, Factor II (prothrombin) G20210A, methylenetetrahydrofolate reductase (*MTHFR*) C677T and A1298C, and plasminogen activator inhibitor (*PAI-1*) 4G/5G. Of a total of 1448 genotypes tested in this study, there were 22 samples that gave different results between the Invader biplex assay and the PCR-based methods. On further testing, 21 were determined to be correctly genotyped by the Invader Assay and only a single discrepancy was resolved in favor of the PCR-based assays. The compiled results demonstrate that the Invader biplex assay provides results more than 99.9% concordant with standard PCR-based techniques and is a rapid and highly accurate alternative to target amplification-based methods. (*J Mol Diagn* 2004, 6:137–144)

the accuracy and precision of the Invader-based assays in the analysis of five loci implicated as risk factors for VT.

The Factor V Leiden (FVL) mutation, an Arg to Gln change at amino acid 506, leads to resistance to cleavage by activated protein C. It is the most common inherited risk factor for venous thrombosis (VT) and is found in 20 to 40% of cases.² Another highly characterized variation, the Factor II G20210A mutation in the 3' untranslated region of the prothrombin gene, is found in fewer cases [18% of thrombosis patients;³], but is viewed as a probable causative factor for VT.^{1,4}

A common variation in the methylenetetrahydrofolate reductase (*MTHFR*) gene, a C to T transition at nucleotide 677 leading to a thermolabile version of the enzyme, has been extensively investigated (reviewed in⁵). A variation in a putative regulatory domain of *MTHFR*, A1298C, results in an enzyme with only 60% of the normal level of catalytic activity.⁶ The C677T mutation, alone and in combination with A1298C, is associated with elevated levels of homocysteine (hcy), known to be a risk factor for VT and other cardiovascular diseases.^{5,7,8}

Elevated levels of PAI-1 are associated with VT,⁹ coronary artery disease,¹⁰ atherothrombotic stroke,¹¹ and various complications during pregnancy.^{12,13} A single-base deletion at nucleotide –675 (4G/5G) in the upstream regulatory region of PAI-1 has been associated with increased levels of PAI-1 protein.¹⁴

Genetic testing has become the standard for determining the presence of these variations.¹ The most widely used methods for determining these genotypes use the polymerase chain reaction (PCR), notably restriction fragment length polymorphism (PCR-RFLP)^{7,15} and allele-specific-PCR (AS-PCR).^{16,17} A novel genotyping method suitable for direct analysis of genomic DNA without target amplification, termed the Invader assay, has been demonstrated to be accurate and effective in detecting FVL.^{18,19} Multi-site studies validated the isothermal microtiter plate-based Invader assay in which wild-type and mutant alleles were assayed individually. In this report, we present results from a multi-site study using an Invader biplex assay in which wild-type and mutant alleles are assayed simultaneously in the same reaction well.

Venous thrombosis (VT) is a multi-factorial disorder that arises from the combined effects of acquired and inherited risk factors [reviewed in¹]. In this study we compare

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Detailed descriptions of the mechanics of the Invader assay have been published elsewhere.^{20–22} An enhancement to the Invader assay, termed the “biplex” format, has recently been introduced for the direct analysis of genomic DNA. The Invader biplex format differs from previous generations of the assay because it enables simultaneous detection of two DNA sequences in a single well. Most often, two alleles of a mutation or two variants of a particular polymorphism are targeted. The Invader biplex assay uses two different discriminatory primary probes, each with a unique 5′ flap, and two different fluorescence resonance energy transfer (FRET) cassettes, each labeled with a spectrally distinct fluorophore.²² By design, the released 5′ flaps will bind only to their respective FRET cassettes to generate a target-specific signal (Figure 1).

This multi-site study designed to evaluate the accuracy and performance of the Invader biplex assay was carried out in seven clinical laboratories. Genotyping results obtained from the Invader biplex assay and AS-PCR or PCR-RFLP were compared for five loci associated with venous thrombosis: Factor V Leiden, prothrombin G2010A, *MTHFR* C677T and A1298C, and *PAI-1* 4G/5G. Of a total of 1448 genotypes determined in this study, the Invader biplex assay made only a single incorrect call. Moreover, the Invader assay made correct calls for 21 samples incorrectly genotyped by the PCR-based methods. Thus, the compiled results demonstrate that the

Invader biplex assay provides results more than 99.9% concordant with standard PCR-based techniques.

Materials and Methods

Sample Collection and Study Design

Samples used in these studies were chosen randomly. Genomic DNA was archived and stored at 4°C or less for no longer than 24 months. All samples were numbered, unlinked, and tested anonymously. No information was available regarding predisposition to thrombosis, related disorders, or other clinical or demographic factors. All samples were extracted from human whole blood or buffy coat from whole blood collected in EDTA or sodium citrate Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ).

Sample Preparation and DNA Quantification

Genomic DNA was extracted using commercially available kits. Each DNA specimen was quantified using either the Molecular Probes (Eugene, OR) PicoGreen dsDNA quantitation method or the Molecular Probes OliGreen method. Samples extracted by the Generation Capture Plate Kit (Gentra Systems, Minneapolis, MN) were quantified using the Molecular Probes Oli-

Invasive structure forms from single-base overlap between Invader® Oligo and WT Probe when hybridized to WT Target DNA.

Mismatch between Mut Probe and WT Target DNA prevents single-base overlap; therefore, no invasive structure is formed.

Figure 1. Invader assay schematic.

Table 1. Protocols for Genotyping Reference Methods

Allele tested	Assay	PCR primers	Positive controls (for AS-PCR) or enzymes (for RFLP)	References or reaction conditions
FVL	AS-PCR	5'-GGGGGACAATTTTCAATATATTTCTTCA-GGCAG-3' (forward) 5'-GGGGGTTCAGGACAAAATACCTGTATT-CCAC-3' (WT reverse) 5'-GGGGGTTCAGGACAAAATACCTGTAT-TCCAT-3' (mutant reverse)	Positive control for the mutant reaction: Prothrombin reverse consensus: 5'-CTC CAA ACT GAT CAA TGA CCT TC-3'. Positive control for the WT reaction: human growth hormone (HGH): HGH 1 5'-CCT TCC CAA CCA TTC CCT TA-3') and HGH2 (5'-CAC GGA TTT CTG TTG TGT TTC-3')	29-31
Prothrombin	AS-PCR	5'-TCT AGA AAC AGT TGC CTG GCA GA-3' (forward consensus) 5'-CAC TGG GAG CAT TGA GGC AC-3' (WT reverse) 5'-CAC TGG GAG CAT TGA GGC AT-3' (mutant reverse)	Positive control for the mutant reaction: Prothrombin reverse consensus: 5'-CTC CAA ACT GAT CAA TGA CCT TC-3' Positive control for the WT reaction: human growth hormone (HGH): HGH1 (5'-CCT TCC CAA CCA TTC CCT TA-3') and HGH2 (5'-CAC GGA TTT CTG TTG TGT TTC-3')	29-31
MTHFR C677T	PCR-RFLP	5'-GCT GAC CTG AAG CAC TTG AAG GAG AAG-3' (forward) 5'-AGG ACG GTG CGG TGA GAG TG-3' (reverse)	Hinf I	7
MTHFR A1298C	PCR-RFLP	5'-CTT TGG GGA GCT GAA GGA CTA CTA C-3' (forward) 5'-CAC TTT GTG ACC ATT CCG GTT TG-3' (reverse)	Mbo II	15, 32
PAI-1	AS-PCR	5'-TGC AGC CAG CCA CGT GAT TGT CTA G-3' (reverse consensus) 5'-GTC TGG ACA CGT GGG GA-3' (forward; 4G) 5'-GTC TGG ACA CGT GGG GG-3' (forward; 5G)	5'-AAG CTT TTA CCA TGG TAA CCC CTG GT-3' (positive control forward)	17, 33
	PCR-RFLP	5'-GAG AGT CTG GAC ACG <u>TCC</u> GG-3' (forward) [the two mutagenic bases are underscored] 5'-AAC AGC CAC AGG GCA TGC A-3' (reverse)	BspEI	Homebrew

Green Quantitation reagents. Only samples containing final DNA concentrations of ≥ 10 ng/ μ l were used in the present study.

AS-PCR, PCR-RFLP, and Sequencing

All sites used PCR-based genotyping reference methods. Each laboratory compared the Invader assay with either the specific PCR-based method currently in use or another widely used method. The primers for each allele as well as the references for the protocols are included in Table 1. Sites one through four determined the FVL and prothrombin genotypes using AS-PCR; sites six and seven determined the PAI-1 genotype by PCR-RFLP; and sites three and five determined the MTHFR C1298T and C677T genotypes by PCR-RFLP. Primers were obtained from Invitrogen (Carlsbad, CA) or Third Wave Technologies, Inc. (Madison, WI).

Samples were sequenced on the ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA) using primers listed in Table 1 and the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Big Dye Terminator with AmpliTaq FS DNA Polymerase).

Mass Spectrometry

An existing subset of samples (100 samples) was characterized for PAI-1 4G/5G by PCR-mass spectrometry

using the MassARRAY system (Sequenom, San Diego, CA). Genomic DNA was extracted by the Generation Capture Plate kit (Gentra Systems) and amplified by PCR to generate a 98-bp fragment of the PAI-1 gene. The unincorporated deoxynucleotides were digested by shrimp alkaline phosphatase (SAP) enzyme. The amplicon was then mixed with hME extension primer, MassEXTEND enzyme (ie, Thermo Sequenase), and an appropriate termination mixture consisting of dNTPs and ddNTPs, to differentially extend the mutant and wild-type alleles. The final extended product was purified by the addition of SpectroCLEAN (cation-exchange beads), and the purified supernatant was spotted onto a SpectroCHIP. Analysis by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) began when air was removed in high vacuum and a laser pulse caused a spontaneous volatilization and ionization of matrix and associated DNA fragments. These gas-phase ions were accelerated through a voltage potential and their time-of-flight (TOF) was recorded. The TOF results were then converted into mass values, which were calculated, recorded, and interpreted for sample genotype.

Invader Assay

All Invader assay components were provided by Third Wave Technologies, Inc. Ten μ l of genomic DNA samples were dispensed into individual wells of 96-well mi-

Table 2. Invader Assay Oligonucleotide Sequences

Factor V Leiden (G1691A) FVL	Invader	5'-TCTAATCTGTAAGAGCAGATCCCTGGACAGGCC-3'
	Invader (1689)	5'-TCTAATCTGTAAGAGCAGATCCCTGGACAGACC-3'
	WT probe	5'-CGCGAGGCCGGAGGAATACAGGTATTTGTCC-Hexanediol-3'
	Mut probe	5'-AGGCCACGGACGAAGGAATACAGGTATTTGTC-Hexanediol-3'
	FRET (WT)	5'-R-TCT-Q-AGCCGGTTTTCCGGCTGAGACGGCCTCGCG-Hexanediol-3'
	FRET (Mut)	5'-F-TCT-Q-AGCCGGTTTTCCGGCTGAGACGTCCGTGGCCT-Hexanediol-3'
	WT target	5'-TCAAGGACAAAATACCTGTATTCCTCGCCTGTCCAGGGATCTGCTCTT- ACAGATTAGAAGTGATTT-3'
	Mut target	5'-TCAAGGACAAAATACCTGTATTCCTTGCCGTGTCCAGGGATCTGCTCT- TACAGATTAGAAGTGATTT-3'
	Invader	5'-TATGGTTCCCAATAAAAGTGACTCTCAGCT-3'
	WT probe	5'-ACGGACGCGGAGGAGCCTCAATGCTACCAG-Hexanediol-3'
Factor II (G20210A) FII	Mut probe	5'-AGGCCACGGACGAAGCCTCAATGCTCC-Hexanediol-3'
	FRET (WT)	5'-R-TCT-Q-TCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-Hexanediol-3'
	FRET (Mut)	5'-F-TCT-Q-AGCCGGTTTTCCGGCTGAGACGTCCGTGGCCT-Hexanediol-3'
	WT target	5'-TAGCACTGGGAGCATTGAGGCTCGCTGAGAGTCACATTTTATTGGGA- ACCATAGTTTTAGAAACACAAAAAT-3'
	Mut target	5'-TAGCACTGGGAGCATTGAGGCTTGCTGAGAGTCACATTTTATTGGGA- CCATAGTTTTAGAAACACAAAAAT-3'
Methylenetetrahydrofolate reductase (677) MTHFR 677	Invader	5'-CAAAGAAAAGCTGCGTGATGATGAAATCGC-3'
	Invader (SIP)	5'-CAAAGAAAAGCTGCGTGATGATGAAATTGC-3'
	WT probe	5'-ACGGACGCGGAGGCTCCCGCAGACAC-Hexanediol-3'
	Mut probe	5'-AGGCCACGGACGACTCCCGCAGACAC-Hexanediol-3'
	FRET (WT)	5'-F-TCT-Q-AGCCGGTTTTCCGGCTGAGACTCCGCGTCCGT-Hexanediol-3'
	FRET (Mut)	5'-R-TCT-Q-TCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-Hexanediol-3'
	WT target	5'-GAAGGTGTCTGCGGGAGCCGATTTTCATCATCACGCAGCTTTTCTTT- GAGG-3'
	Mut target	5'-GAAGGTGTCTGCGGGAGTCGATTTTCATCATCACGCAGCTTTTCTTT- GAGG-3'
	Invader	5'-CCCCGAGAGGTAAAGAACAAAGACTTCAAAGACACTTA-3'
	WT probe	5'-AGGCCACGGACGTCTTCACTGGTCAGCT-Hexanediol-3'
Methylenetetrahydrofolate reductase (1298) MTHFR 1298	Mut probe	5'-CGCGCCGAGGGCTTCACTGGTCAGC-Hexanediol-3'
	FRET (WT)	5'-F-ACT-Q-AGCCGGTTTTCCGGCTGAGTCGTCCGTGGCCT-Hexanediol-3'
	FRET (Mut)	5'-R-TCT-Q-TCGGCCTTTTGGCCGAGAGACCTCGGCGCG-Hexanediol-3'
	WT target	5'-GGAGCTGACCAGTGAAGAAAGTGTCTTTGAAGTCTTTGTTCTTTACCT- CTCGGGATTT-3'
	Mut target	5'-GGAGCTGACCAGTGAAGCAAGTGTCTTTGAAGTCTTTGTTCTTTACCT- CTCGGGATTT-3'
Plasminogen Activator Inhibitor-1 (PAI-1-675 4G/5G) PAI-1 4G/5G	Invader	5'-GCACAGAGAGAGTCTGGACACGTGGGGT-3'
	WT probe (5G)	5'-AGTCGTCCGACAGAGTCAGCCGTGTATCA-Hexanediol-3'
	Mut probe (4G)	5'-ACGGACGCGGAGAGTCAGCCGTGTATCA-Hexanediol-3'
	FRET F	5'-F-TCT-Q-AGCCGGTTTTCCGGCTGAGATGTCGGACGAGCT-Hexanediol-3'
	FRET R	5'-R-TCT-Q-TCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-Hexanediol-3'
	WT target	5'-CCGATGATACACGGCTGACTCCCCACGTGTCCAGACTCTCTCTGTG- CCCC-3'
	Mut target	5'-CCGATGATACACGGCTGACTCCCCACGTGTCCAGACTCTCTCTGTG- GCCCC-3'

crotiler plates and denatured at 95°C for 5 minutes. Ten μ l of a reaction mix containing the appropriate probes/Invader oligo/MgCl₂ mix were added, and reactions were overlaid with 20 μ l of molecular biology-grade mineral oil (Sigma Chemicals, St. Louis, MO). Each 20- μ l reaction contained 40 ng Cleavase enzyme, 3.5% PEG 8000, 2% glycerol, 0.06% NP 40, 0.06% Tween 20, 12 μ g/ml BSA, 0.25 μ mol/L each of F (FAM) dye and R (Redmond Red) dye (Epoch Biosciences, Redmond, WA) FRET cassettes, 8 mmol/L MgCl₂, 0.5 μ mol/L of each allele-specific probe, and 0.05 μ mol/L Invader oligo. The sequences of the Invader oligos, wild-type and mutant primary probes, wild-type and mutant FRET probes and corresponding synthetic secondary targets are presented in Table 2. Following the reagent dispensing, plates were incubated at 63°C for 4 hours in a PTC-100 thermal cycler (MJ Research, Waltham, MA). Fluorescence was measured directly at the end of the incubation period using a CytoFluor 4000 fluorescence plate reader

(Applied Biosystems, Foster City, CA). The settings used were: 485/20 nm excitation/bandwidth and 530/25 nm emission/bandwidth for F dye detection, and 560/20 nm excitation/bandwidth and 620/40 nm emission/bandwidth for R dye detection. The instrument gain was set for each dye so that the No Target Blank produced between 100 to 200 absolute fluorescence units (AFUs).

Each microtiter plate contained synthetic DNA controls provided by Third Wave Technologies, Inc. for each genotype, ie, homozygous wild-type, heterozygous and homozygous mutant, as well as a No Target Blank.

Data Analysis

Calculation of Ratios and Guidelines for Interpretation

Data were analyzed using a Microsoft Excel-based spreadsheet (Microsoft, Redmond, WA). For each allele of a given polymorphism, the net signal/background, or

Table 3. Data Analysis

Ratio	Mut FOZ*	WT FOZ*	Genotype
≥5.0	≥2.0	≥2.0	WT
>2.0 to <5.0 [†]	—	—	Equivocal (EQ1)
≥0.5 to ≤2.0 [†]	≥2.0	≥2.0	Het
>0.2 to <0.5 [†]	—	—	Equivocal (EQ2)
≤0.2	≥2.0	≥0.2	Mut

* For PAI-1, the FOZ criteria were lowered to 1.75.

[†] For MTHFR, the boundaries were set at 0.3 and 3.0 due to differences in the amounts of signal generated by the Invader assays.

Net Fold Over Zero (FOZ – 1), values were calculated as follows for the signal obtained with each dye:

$$\text{FOZ} = \frac{\text{Raw counts from sample}}{\text{Raw counts from No Target Blank}}$$

The two FOZ values (ie, wildtype and mutant) for each sample were used to calculate the WT:Mut ratio as follows:

$$\text{Ratio} = \frac{(\text{Net WT FOZ})}{(\text{Net Mut FOZ})}$$

where net FOZ = FOZ – 1

In cases for which the net FOZ was ≤0.04, the value was set to 0.04 to calculate the ratio to avoid division by zero as described elsewhere.²³

Run Acceptance Criteria

In order for a sample run, ie, a microtiter plate containing controls and samples to be deemed valid, the FOZ ratios of the controls had to be within specific ranges. The FOZ ratio criteria were 5 or greater for the normal control, between 0.5 and 2 for the heterozygous control, and 0.2 or lower for the homozygous mutant control (except for *MTHFR*, where the FOZ ratios had to be between 0.3 and 3 for the heterozygous control). The FOZ values for the controls also had to meet minimum FOZ criteria (1.5 and 0.2 for *FII*, *FVL*, *MTHFR*, or 1.75 and 0.2 for *PAI-1*). Runs failing these criteria were deemed invalid and were repeated.

Sample Acceptance Criteria

Samples included in the study had to meet the following two criteria.

1. The ratio of net FOZ had to fall into the WT, Het (heterozygous mutation) or Mut (homozygous mutation) range; and

2. The FOZ value for each signal of the sample had to be greater than or equal to the values shown in Table 3

Results falling into either equivocal range as well as those failing the FOZ criteria were deemed invalid and subject to re-analysis; samples that met the ratio and FOZ criteria on re-testing were included in the study.

Results

Comparison of PCR and Biplex Invader Assay Results

Genotypes were generated from 1475 samples using the biplex Invader assay and AS-PCR, PCR-RFLP, or PCR-mass spectrometry. Of these samples, 1448, or 98.2%, were suitable for inclusion in the study. Results from a previous study demonstrated that neither the sample type (ie, buffy coat *versus* whole blood) nor the choice of extraction method affects the accuracy of the Invader assay.¹⁸

The results comparing the Invader assay to a reference method from each center are presented in Table 4. Samples were analyzed in separate runs, each comprising four controls and approximately 20 samples. The overall concordance rate for the combined results was greater than 99.9% (1447 of 1448 correct calls). The Invader assay failed to assign a genotype correctly in only one case. The sample was homozygous wildtype but was incorrectly called a heterozygote in the initial Invader assay. The Invader assay yielded the correct result on repetition. DNA sequencing revealed that 21 samples that were initially discordant were correctly genotyped by the Invader assay, but were miscalled by the PCR-based reference method. For the PAI-1 assay, six of the seven samples called incorrectly by PCR were genotyped by the PCR-mass spectrometry assay.

Twenty-five samples yielded Invader assay results that fell into one of the two equivocal ranges established to enhance the discrimination of heterozygotes from either homozygote. Of these samples, 20 had adequate levels of DNA to support re-testing; 17 yielded valid assay results on re-testing, and three samples remained equivocal and thus could not be typed. Another 16 samples were initially invalid because they failed the net signal/background or FOZ criteria, meaning that they did not

Table 4. Summary of Invader Genotyping Results

Polymorphism	Wild type	Heterozygote	Mutant	Invader assay totals	PCR-based assay totals
Factor V Leiden	300/301 (99.7%)	58/58 (100.0%)	9/9 (100.0%)	367/368 (99.7%)	368/368 (100%)
Prothrombin	338/338 (100%)	38/38 (100%)	1/1 (100%)	377/377 (100%)	363/377 (96.3%)
MTHFR C677T	87/87 (100%)	83/83 (100%)	25/25 (100%)	195/195 (100%)	195/195 (100%)
MTHFR A1298C	97/97 (100%)	85/85 (100%)	18/18 (100%)	200/200 (100%)	200/200 (100%)
PAI-1	64/64 (100%)	174/174 (100%)	70/70 (100%)	308/308 (100%)	301/308 (97.7%)
Overall accuracy				1,447/1,448 = 99.93%	1,427/1,448 = 98.5%

Figure 2. Graph of MTHFR A1298C wild-type: mutant ratios. Ratios obtained from Invader assay analysis of 200 samples are presented. The different genotype and equivocal zones are indicated.

generate sufficient signal to produce a valid result. The most likely cause for FOZ failure is inadequate DNA concentration. Only nine of these samples were re-tested; four of these samples passed the FOZ sample acceptance criteria. Seven samples initially obtained for the study gave valid results in the Invader assay, but could not be amplified by PCR. Without a second genotype with which to compare the Invader call, these samples could not be included in the study.

Figure 2 graphically presents the distribution of ratios for the *MTHFR* A1298C assay to illustrate genotype differentiation. As with the previous generation of the Invader assay, all three genotypes are clearly separated into distinct ranges of signal ratios. Previous reports describing Invader-based FVL genotyping included only a single equivocal zone, between the zones for heterozygotes and wild-type homozygotes.^{18,19} A second equivocal zone has been added between heterozygotes and mutant homozygotes because, unlike for *FVL*, mutant alleles are frequently encountered at the *MTHFR* and *PAI-1* loci.

The sample populations included for this study were selected from archived samples by each site. No effort was made to ensure that the samples represented any particular population.

Discussion

Despite the proliferation of commercially available methods for detecting single nucleotide polymorphisms (SNPs), clinical and reference laboratories continue to rely heavily on labor-intensive, PCR-based methods such as AS-PCR and PCR-RFLP for many routine genotyping tests.

The results of this study highlight the suitability of the Invader biplex assay, in terms of both its accuracy and its ease-of-use, for genotyping diverse alleles implicated in the pre-disposition to venous thrombosis. Previous studies indicated that the second generation, monoplex Invader assay was a highly accurate alternative to AS-PCR for detecting the *FVL* mutation; initial concordance be-

tween the Invader assay and AS-PCR was 99.7% in a study conducted in a single laboratory¹⁹ and 99.5% in a multi-site study.¹⁸ In the present multi-site study, the biplex assay yielded an accuracy rate of 99.7% (367 of 368 correct calls) for *FVL*. Overall, the Invader biplex assay was 99.9% (1447 of 1448 correct calls) accurate when multiple alleles were tested at multiple sites. Initially, 22 samples gave discordant results when the outcomes of the Invader and reference assays were compared; however, in all but one case, the discordance arose from incorrect initial calls by the PCR-based reference methods. The one sample that was initially identified as discordant by Invader was correctly called by Invader on repetition. Specifically, Invader gave a heterozygote determination to a homozygous wild-type sample on initial testing, but made the correct genotype determination when the sample was repeated. There was no obvious cause for the original discrepancy. Thus, the Invader assay is a highly accurate genotyping method (1447 of 1448 correct calls, or 99.93%) compared to the PCR-based reference methods (1427 of 1448 correct calls, or 98.5%). The primary advantage of the new Invader biplex assay format is its ability to detect the three possible genotypes for a given sample in a single well. The inclusion of two Invader assays in each well provides a *de facto*-positive internal control; the biplex format obviates the possibility of mistaking a heterozygote for a homozygote through failure to add template or reagents to a reaction. Absence of signal, arising from improper reaction set-up, lack of genomic DNA sample or inhibition, indicates that the sample must be reanalyzed. Furthermore, the biplex assay utilizes a new *Cleavase* enzyme that, coupled with improved design of the FRET cassettes, results in increased specific signal per nanogram of genomic DNA sample and lower non-specific background signal. The addition of a second dye that is spectrally distinct from the first permits two signals to be read from the same well simultaneously without cross-talk. The incorporation of a non-fluorescent "dark" quencher also reduces fluorescence background.

The Invader biplex assay, like the monoplex version that preceded it, is completely homogeneous; that is, it requires no separation steps. Recent improvements have reduced the number of reagent additions and eliminated the need to transfer reactions before fluorescence detection.²³ While it is suitable for robotic automation, the assay can also be performed manually, as the present study demonstrates, with no loss of accuracy. The seven participant sites in the present study spanned a broad range of technical expertise *vis a vis* molecular testing in general and the Invader assay in particular. At one end of the spectrum, one of the participating laboratories performs no molecular testing other than Invader assays, while at the other end are laboratories that offer a complete menu of molecular tests, including some based on the Invader assay.

Although the Invader assay requires more genomic DNA (100 ng) than PCR-based assays, the quantity of DNA is usually not limiting for individual SNP assays. Qiagen's standard blood and body fluids extraction protocol typically yields 6 μ g of DNA (www.qiagen.com),

and the Puregene DNA Purification kit yields 1.5 to 5 μ g of DNA from 100 μ l of whole blood (www.gentra.com). By using an assay that requires more genomic DNA but does not use PCR, laboratories can save time as well as costs by avoiding PCR amplicon contamination problems and precautions.

The suitability of the Invader assay for both ultra high-throughput²⁴ and low- to medium-throughput studies^{18,19,25–27} is well documented. In addition to meeting the stringent demands for accuracy required in such settings, the Invader assay is readily adapted to diverse throughput demands. Invader assays have been developed and validated for more than 120,000 unique sequences for use at genome centers,²⁴ pharmaceutical companies, and research laboratories worldwide.^{24,27,28} For the small-scale studies described here, the only equipment needed is a thermal cycler and a fluorescence plate reader. This study, the first to apply the Invader biplex assay format to clinical samples in multiple laboratories, demonstrates that the biplex format is highly accurate and suitable for routine clinical genetic testing.

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