A Multi-Site Study for Detection of the Factor V (Leiden) Mutation from Genomic DNA Using a Homogeneous Invader Microtiter Plate Fluorescence Resonance Energy Transfer (FRET) Assay

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The goal of this multicenter study was to evaluate the second-generation Invader technology for detecting the factor V (Leiden) mutation directly from genomic DNA of different sample types. Invader assay results were compared with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or allele-specific PCR (AS-PCR) analysis. The Invader assay is a PCR-independent methodology that uses a microtiter plate format. In the assay, a specific upstream Invader oligonucleotide and a downstream probe hybridize in tandem to a complementary DNA template and form a partially overlapping structure. The Cleavase VIII enzyme recognizes and cuts this structure to release the 5′ flap of the probe. This flap then serves as an Invader oligonucleotide to direct cleavage of a fluorescence resonance energy transfer (FRET) probe in a second invasive cleavage reaction. Cleavage of this FRET probe results in the generation of a fluorescent signal. The results of the Invader assay were 99.5% concordant with the PCR-based methods. Of the 372 samples tested once, only two gave discordant results (one from operator error and one from unknown causes), but were concordant on retesting. These results indicate that a simple microtiter plate-based Invader assay can reliably genotype clinical patient samples for the factor V (Leiden) point mutation directly from genomic DNA without prior target amplification. (J Mol Diag 2000, 2:97–104)

The most common known genetic risk factor found in patients with deep venous thrombosis is a single G-to-A base change at nucleotide 1691 (G1691A), termed the factor V (Leiden) mutation (FVL). The mutation appears in 20 to 60% of patients examined for a predisposition to deep venous thrombosis and occurs in 2 to 5% of the general population. In individuals who have the FVL mutation, the activated form of factor V (factor Va) is resistant to cleavage by activated protein C (APC), a component of the natural anticoagulant system. The mutation causes the substitution of an arginine for a glutamine at residue 506 (R506Q) in the protein, altering one of the three APC cleavage sites on factor Va, thereby increasing factor Va’s resistance to cleavage, and produces a phenotype termed APC resistance. Individuals heterozygous for the mutation have a three- to eightfold greater risk for thrombosis, and homozygotes have nearly a 100-fold greater risk, compared to individuals without the mutation. Evaluation for the presence of the FVL mutation is important for establishing whether a patient has a predisposition to thrombosis, for designing appropriate patient management protocols, and for identifying individuals and families who face increased risk for future thrombotic events. A functional test for APC resistance can be used to screen for the phenotype conveyed by this mutation. However, the designation of an APC resistance assay result as abnormal is based on statistical methods. Further, acquired conditions due to lupus anticoagulant or warfarin and heparin anticoagulant therapies can interfere with the results from the originally described APC resistance test, unless modifications are made. Therefore, a genetic assay has been recommended to confirm positive results and, most important, to conclusively distinguish heterozygotes from homozygotes. Typical confirmatory tests for the mutation use a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique or allele-specific PCR (AS-PCR). The availability of a cost-effective and rapid microtiter plate-based molecular test that requires no special containment and is compatible with high-throughput automation would enable many more clinical laboratories to test directly for the genetic mutation.

A non-PCR-dependent methodology, Invader technology, has been developed (Third Wave Technologies, Inc., Madison, Wis.).
Inc., Madison, WI) and applied to detect the FVL and other mutations.12–15 The Invader assay, described schematically in Figure 1, can simultaneously provide linear signal amplification for target quantitation and discriminate single nucleotide changes, such as the FVL mutation, directly from genomic DNA without the need for prior target amplification. A microtiter plate-based format makes the assay adaptable for small or high-throughput clinical laboratory settings.

Invader technology relies on Cleavase enzymes, a class of naturally occurring and engineered nucleases that cleave unpaired regions (also called flaps) of DNA, which form when the 5’ end of a DNA sequence overlaps the hybridization site of the 3’ end of an upstream oligonucleotide by at least 1 bp. Substrate recognition of the overlapping, or invasive, structure is the basis for discrimination of single-basepair changes. Detection is accomplished through a fluorescence resonance energy transfer (FRET) mechanism.12 FRET occurs when energy from an excited donor fluorophore is transferred to a nearby acceptor dye. When the acceptor is physically near the donor, it quenches the donor’s fluorescence emissions. During the assay, when cleavage separates the donor fluorophore from the acceptor dye, quenching is minimized and a fluorescent signal is generated.

The first-generation Invader assay has been reported previously13; the studies described in this paper use the second generation of Invader technology. The design of the second-generation Invader assay differs from the first generation in four respects, offering several advantages in assay performance and data collection. First, the second-generation Invader assay uses a simple, isothermal format. In contrast, the first-generation assay required two different temperatures and separate additions for the primary and secondary reactions. Second, both the cleaved and uncleaved primary probes could bind stably to the secondary target in the first-generation assay. Arrestor oligonucleotides were added to bind excess uncleaved primary probes. In the second-generation assay, uncleaved primary probes do not bind stably to the FRET probe, so arrestors are not needed. Third, the second-generation Invader assay employs a single synthetic oligonucleotide that combines both target and a fluorescent-labeled signal probe in the second cleavage reaction, whereas the first-generation Invader assay used two separate synthetic oligonucleotides (a secondary target and a secondary fluorescent-labeled signal probe) to generate a fluorescent signal after cleavage. The identical FRET probe can be used with different analytes, for two reasons. The identity of the 3’ terminal base of the
primary or secondary Invader oligo does not affect the efficiency of the reaction, and the target-independent 5’ flap of the primary probe complements only the FRET probe. These design features enable the use of a “universal” secondary system with second-generation Invader technology. Fourth, the second-generation Invader assay permits direct reading of assay results, although this method was not used in the studies reported here.

Figure 1 shows a schematic of the Invader assay, illustrating how it detects the DNA mutation of interest. In the Invader assay, two oligonucleotides, a wild-type (WT) or mutant (Mut) probe oligonucleotide and an Invader oligonucleotide, hybridize in tandem to a specific region of DNA to generate the precise overlapping structure recognized by the Cleavase VIII enzyme. This structure includes an unpaired flap on the 5’ end of the downstream probe (WT or Mut) oligonucleotide. Once the proper structure is formed (Figure 1A), the Cleavase VIII enzyme cleaves the 5’ flap, releasing it as a target-specific product. The released flap now serves as an Invader oligonucleotide in a second invasive cleavage reaction on a labeled, synthetic target, the FRET probe (Figure 1A). The FRET probe is 5’-end labeled with a donor fluorophore, fluorescein (F), that is quenched by Cy3, an acceptor dye (Q). Cleavage separates the fluorescein and the quencher and produces the 5’-fluo-

rescein-labeled product that is detected with a fluorescence microtiter plate reader. If the precise invasive structure is not formed in the first step, as in the case of Mut target (Figure 1B), cleavage will not occur in either the primary or the secondary reaction, and no or a minimal fluorescent signal will be generated. The identity of the 3’ terminal base of an Invader oligo does not affect the efficiency of the reaction. As a result, the identical primary Invader oligonucleotide can be used with both wild-type and mutant targets. Further, the cleavage products from both the mutant and the wild-type primary reactions can be used with the same secondary system.

The target DNA is the limiting component in the first invasive cleavage, and Invader and probe oligonucleotides are supplied in molar excess. Reactions are carried out at temperatures near the melting temperature \(T_{m}\) of the analyte-specific region of the WT or Mut probe; so the probes turn over without temperature cycling to produce many signals per target. Similarly, the released 5’ flap from the primary reaction is limiting in the second invasive cleavage reaction relative to the molar concentration of the FRET probe. At the temperature of the assay, each target-specific product of the primary reaction can enable the cleavage of many FRET probes in the secondary reaction to further amplify the target-specific signal. The fluorescent signal from the composite reaction accumulates linearly with respect to the target DNA. Because these two coupled cleavage reactions occur simultaneously, they can produce \(10^6\) to \(10^7\) labeled cleavage products per target sequence per hour, producing at least a million-fold increase in signal.

A multisite study was performed in clinical laboratories to compare the genotyping for FVL of patient samples with the Invader assay and either PCR-RFLP or AS-PCR. The data show that the Invader method provides results concordant with the standard techniques in more than 99.5% of the samples on initial testing.

### Materials and Methods

#### Patient Samples and Study Design

Individuals chosen for this study comprised patients presenting with signs or symptoms of thrombosis, patients at risk for a thrombotic event as a result of clinical or environmental risk factors, and patients with a familial history suggesting a predisposition for thrombosis. All samples were numbered, unlinked, and tested anonymously. Each sample was tested once.

#### Sample Preparation

Genomic DNA was extracted either from the buffy coat or directly from whole blood treated with sodium citrate, EDTA, or acid citrate dextrose (ACD). Different extraction techniques were used at the various study sites. Table 1 summarizes the specimen type and method used at each site. All archived samples (157 samples, 42%) used in this study were stored for no longer than 24 months at −20°C or colder.

#### DNA Quantitation

DNA preparations were quantitated using the PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions, as previously described. At site one, DNA was also quantified spectrophotometrically by measuring the absorbance at 260 nm on a Hitachi U2000 spectrophotometer (Hitachi, Ltd., Tokyo, Japan) or a SPECTRAmax Plus (Molecular Devices, Sunnyvale, CA). The study required a minimum of 70 ng of genomic DNA per reaction.

#### AS-PCR and PCR-RFLP

AS-PCR-based genotyping, conducted at sites one and three, was performed essentially as previously described. Site one used 5’-GGGGGACAATTT-3’ for the factor V forward consensus primer and either 5’-GGGGTCAAGGA-CAAAATACCTGTATTCCAC-3’ for the wild-type factor V...
reverse primer or 5'-GGGGGTCAAGGACAAATACCTG- TATTCCT-3' for the mutant FVL reverse primer, along with the appropriate primers for methylenetetrahydrofolate reductase and prothrombin genotyping. Because most individuals are homozygous wild-type for these three alleles, the mutant reaction used an antisense prothrombin consensus primer as an internal positive control. Site three used 5'-AGGAACACACCCTATGTC-3' for the factor V forward consensus primer, either 5'-GGGAAATATCTG- TATTCAC-3' for the wild-type factor V reverse primer or 5'-GGACAAAAATACCTGATTCCTC-3' for the mutant FVL reverse primer. Two primers for the human growth hormone (HGH) gene were used as internal positive controls: HGH1 (5'-TGCCCTCCACACCATTCCCAA-3') and HGH2 (5'-CCACTACGATTTCTGTTGCTT-3'). Site two used PCR-RFLP, using the loss of an Mnl1 restriction site caused by the G-to-A transition at nucleotide 1691 of the factor V gene. The primers used were 5'-TGCCCCAGTGCTTAACAAGACCA-3' (sense primer, nt 1581 to 1602) and 5'-TGTTATCACACTGCTGCTAA-3' (anti-sense primer, nt −146 to −127).

**Invader Assay**

All components of the Invader assay were provided by Third Wave Technologies, Inc. (Madison, WI). Five microliters (5 μl) of Target/Primary Invader Reaction Mix (PEG 8000, MOPS pH 7.5, the antimicrobial preservative ProClin 300 (Supelco, Bellefonte, PA) and 0.1 μmol/L Invader oligonucleotide) were dispensed into 96-well microplates. Ten microliters of patient DNA sample at a DNA concentration of at least 7 ng/μl or the appropriate control were added with mixing. For each run, wild-type, heterozygous, and mutant target controls, and no target blanks (negative controls) were tested. The heterozygous and mutant synthetic target controls were oligonucleotides. Mixtures were overlaid with 20 μl clear Chill-Out 14°C to 1°C. (MJ Research, Watertown, MA). The no target blanks (yeast tRNA) were used to determine probe-specific background values for each. The oligonucleotide sequences for the target site on the wild-type and Factor V Leiden mutant sequences were 3'-AGATTAGACATTTCGTCTAGGGACCTC- CTCGTGCGCTCTCATATGCTCATAAAACAGGA-5' and 3' AGATTAGACATTTCGTCTAGGGACCTGTCCGGTCCTC- TTATGCTCATAAAAACAGGA-5', respectively; the C-to-T change is shown in bold. Samples were denatured by incubation at 95°C for 5 minutes. in an MJ Model PTC-100 thermocycler (MJ Research), a GeneAmp PCR System 9600 Thermal cycler (Perkin-Elmer, Norwalk, CT), or a GeneAmp PCR System 9700 Thermal cycler (Perkin-Elmer) before the assay. The temperature was lowered to 63°C and 5 μl of the appropriate Cleavase enzyme/ Mg²⁺/probe reaction mix was added to each well, giving a final reaction volume of 20 μl with final concentrations of 3.2% PEG 8000, 2.5 mmol/L MOPS, 0.025% ProClin 300 (Supelco), 25 nmol/L Invader oligonucleotide, 0.5 μmol/L wild-type or mutant probe, 0.5 μmol/L FRET probe, 7.5 mmol/L MgCl₂, and 200 ng Cleavase VIII enzyme. The microplate was incubated at 63°C for 4 hours, and the reaction was stopped by adding 100 μl stop solution (10 mmol/L EDTA).

One hundred microliters of each reaction were transferred to a Costar solid black 96-well microtiter plate (Corning, Inc., Acton, MA) and read either in a CytoFluor Series 4000 fluorescence multiwell plate reader (PerSeptive Biosystems, Framingham, MA) using the following settings: excitation: 485/20 nm (wavelength/bandwidth); emission: 530/25 nm (wavelength/bandwidth); gain: approximately 40–65 (to yield no target counts of 150–200); reads/well: 30; set temp: 25°C; and plate type: CoStar 96-well or CytoPlate 96-well, or in a Dynex Fluorite 1000 fluorescence multiwell plate reader (Dynex Technologies, Inc., Chantilly, VA) using the following settings: excitation: 485 nm; emission: 530 nm; gain: 3–4V; and plate type: standard 96-well.

**Data Analysis**

Net counts for each probe signal were calculated by subtracting the appropriate no target blank signal (background) for the run from the corresponding probe signal. Determination of genotype was based on the ratio of the net counts (wild-type reaction) to the net counts (mutant reaction) as described below:

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\text{Ratio} = \frac{\text{Net counts wild-type probe}}{\text{Net counts mutant probe}}
\]

In cases where the net counts were equal to or less than zero, the net counts were set equal to 1, to rank all ratios as positive numbers and to avoid division by zero. The guidelines shown below were used to interpret Invader assay results. Although the cutoff value for the ratios (Table 1) may be less stringent and the ranges used may be broader than necessary at a particular site, use of these guidelines accommodates differences among sample preparation methods, sample dispensing methods, and plate readers at different sites, any of which could introduce systematic error.

**Sample and Assay Inclusion Criteria**

All assayed samples contained at least 70 ng DNA/asay. Acceptable signal strength was defined as net wild-type or net mutant counts greater than 30. Any sample that did not meet this criterion was re-assayed. Any sample that had acceptable signal strength but yielded a ratio in the equivocal range was re-assayed. If any controls yielded an incorrect genotype determination, the assay was considered invalid and the run was repeated.

**Results**

**Comparison of PCR and Invader Assay Results**

A total of 372 samples were analyzed by the Invader assay and compared to the results from PCR-RFLP or AS-PCR analysis (Table 2). The samples were analyzed in 17 runs: 5 at the Southeast Wisconsin Blood Center and 6 each at the University of New Mexico and the
University of Florida. The Invader assays and PCR-RFLP or AS-PCR yielded concordant results in all but two samples, for an overall concordance rate of 99.5%. Of the two discordant samples, one was a heterozygous incorrectly called homozygous mutant. That mistake arose from a documented operator error. The operator checked the levels in the wells and saw that the wild-type well had less volume than the other wells, and concluded that no DNA was delivered into the well during assay setup. The second error resulted in a heterozygote being called wild-type. We do not know the cause of that error, but, based on the signal data, we speculate that either no DNA or insufficient DNA was added to the mutant probe reaction. On retesting, both samples yielded results concordant with the standard technique.

Table 1 clearly demonstrates that the Invader assay can determine the various FVL genotypes and distinguish among them using the guidelines shown above for calculated net count ratios. To prevent uncertainty in discriminating between wild-type and heterozygous genotypes, a narrow equivocal range was set. Any sample producing results that fell in the equivocal range (greater than or equal to 3, but less than 5) was retested. In this study, of the 372 samples tested, only one (0.27%), was classified as equivocal (a ratio of 4.8) and on retesting was correctly identified as wild-type (a ratio of 26).

![Figure 2. Distribution of ratios from the Invader assay results at the study sites for wild-type, heterozygous, and mutant genotypes, as determined by PCR-RFLP or AS-PCR. Note that the scale for the ordinate is logarithmic.](image-url)
Two samples did not meet sample inclusion criteria and had to be rerun for inclusion in the study. A sample from site one had unacceptably low signal strength. The retest produced net counts greater than 30 and yielded results concordant with the standard technique. A sample from site two produced equivocal results (a ratio >3 but <5), and the retest yielded results concordant with PCR. Two runs were invalid because of incorrect calls of the synthetic target controls. The runs were valid on repeat.

The accuracy of the Invader assay is illustrated by Figure 2, which graphically depicts the actual distribution of the data points. All three classes (homozygous mutant, heterozygote, and wild-type) are clearly separated, with the exception of the single wild-type sample that fell into the equivocal range and that was resolved on retesting (discussed above). The upper limit in the net count ratios (shown above) for classifying a sample as homozygous mutant was 0.25, but no homozygous mutant sample yielded a ratio more than half that value. The upper and lower ratio limits for classifying a sample as heterozygous were less than or equal to 0.25 to less than 3.0, but the actual range of ratios for samples (0.49–2.19) was well within those limits. The lower cutoff value for classifying a sample as wild-type was 5, but no wild-type sample in this study produced a ratio lower than 7.8. Ratios greater than 10 were noted in 99% (313/316) of wild-type samples.

**Discussion**

FVL genotyping is a commonly performed molecular diagnostic test. Although PCR-RFLP and AS-PCR are standardized assays with reliable performance, the techniques can be labor-intensive and require expertise as well as special facilities and procedures to prevent contamination, limiting their general applicability in diagnostic laboratories. As a result, many laboratories use a functional clotting assay for resistance to APC as a screening or surrogate test for FVL. The clotting assay compares well with PCR-RFLP and AS-PCR, if a second-generation APC resistance assay is used. Although the functional APC resistance test can reliably detect protein C resistance, follow-up molecular testing generally is recommended in samples showing borderline or abnormal results, to rule out other causes of functional APC resistance. Furthermore, molecular testing can simultaneously confirm a patient’s zygosity, which is critical, as the thrombotic risk for homozygotes is more than 10-fold greater than the risk for factor V heterozygotes. In addition, the current recommendations for anticoagulant therapy after deep venous thrombosis differ for heterozygotes and homozygotes. Not all clinical laboratories have on-site facilities or the expertise for PCR-RFLP or AS-PCR genotyping, and therefore they must refer samples to outside laboratories. This practice can add significant time and expense to the patient evaluation. The availability of a rapid, simple assay with a streamlined workflow would allow many clinical laboratories to use the more definitive molecular assay to detect the FVL mutation. The Invader assay offers such an option. The Invader assay requires few reagent additions and is run isothermally in a microwell plate, simplifying equipment needs and reducing lab space requirements and hands-on time. Although thermocyclers were used in this study, the Invader assay has been run successfully in heat blocks and water baths.

The ease of integrating Invader technology smoothly into routine clinical laboratory workflow makes it an attractive alternative to target amplification methods, such as PCR, which require separation of pre- and post-PCR areas and special procedures to minimize contamination. In PCR-RFLP, each target must be amplified separately in individual reactions. In addition, different targets may need different endonuclease enzymes for digestion. Further, both PCR-RFLP and AS-PCR require gel electrophoresis for endpoint determination. Each of these steps can affect the hands-on time and the time to reportable results.

In contrast, the uniform reaction conditions used in the Invader assay permit detection of multiple targets from the same sample, simply by using different wells of the microtiter plate for each analyte-specific probe set. This procedure is performed directly on genomic DNA and accumulates signal molecules only when the specific target DNA sequence is present, without the risk of carryover contamination associated with PCR. To produce a strong signal, the Invader assay requires at least 70 ng of DNA template per reaction. We did not investigate the sensitivity of this assay to lower amounts of DNA template, but anecdotally, runs performed outside this study with less DNA template produce considerably less signal.

Notably, the Invader assay requires no special facilities and the training time is shorter than that required for PCR. The FVL Invader assay offers an easy setup and involves a series of simple pipetting steps followed by reading the plate on a fluorescence microtiter plate reader. Total hands-on times for the Invader assay and AS-PCR are about 1 to 1.25 hours, and about 2 hours for PCR-RFLP. In practice, results are available from Invader assays within 1 working day. Typically, PCR-RFLP results are available in 2 working days.

Invader assays have a distinct specificity advantage over assays based solely on hybridization (eg, AS-PCR amplification, chip arrays, dot blots). As shown in this study, Invader technology provides greater than 99% accuracy in a simple and reliable platform. The combination of site-specific hybridization and structure-specific enzymatic cleavage confers remarkable specificity and robustness to the assay. In addition, this requirement limits cross-reactivity arising from nonspecific hybridization and obviates the need for stringent hybridization conditions. The homogeneous reaction conditions used in the Invader assay make this assay format well suited to automation. We did not correlate our genotypic results with phenotypic APC resistance assays or clinical parameters, because the laboratories in this study serve as reference laboratories and those data were not available in our study. The samples studied here were referred to reference laboratories, which likely accounts for the
slightly lower rate of positive FVL mutations in our study than would be expected in a true thrombotic population.

Additional probe sets for genetic hemostatic risk factors, such as the prothrombin (factor II) G20210A\textsuperscript{20} and the MTHFR C677T mutations,\textsuperscript{21,22} are in final development. Assays using these probe sets will be run under the same reaction conditions as the FVL assay. This means that assays for all three risk factors can be performed in the same run, simply by using different wells of the microtiter plate for each analyte-specific probe set. Invader technology is designed to use a universal secondary system. The 5′-flap released from any probe oligonucleotide in the primary cleavage reaction is identical and complements the uniform FRET probe. Consequently, the secondary cleavage reaction can function with almost any primary probe oligonucleotide. Employing the same FRET probe to characterize different targets simplifies assay development, streamlines workflow, and reduces costs. Invader technology has already been used for simultaneous quantitation of the hepatitis B virus (HBV) viral load and detection of the L515 M/L mutation for lamivudine resistance from the same specimens.\textsuperscript{23}

Two samples of the 372 tested gave results discordant with the PCR-RFLP method. Retesting both Invader and PCR-RFLP yielded results concordant with the original PCR-RFLP results. In one case, a heterozygous sample called homozygous mutant by the Invader assay, the discrepancy was due to a failure to add genomic DNA to the well. In the second case, a heterozygous sample called wild-type, there was no obvious cause for the discrepancy. In this second case, the ratio of net wild-type probe counts to net mutation probe counts was 5.04 on the initial run and 0.76 on the second run. Thus, the ratio on the erroneous run was just above the threshold used in the study for identifying a wild-type genotype.

Laboratories designing strategies for using the Invader technology for clinical testing may consider several means to improve accuracy from the 99.5% accuracy that we experienced. One option would be to perform all assays in duplicate, resolving discrepancies with a third run or an independent method. Less expensive measures can also be devised that will address the types of discordant results experienced in this study. To this end, authors at one of the sites in this study (T. M. W. and C. M.) have adopted the following measures. First, operators are reminded to carefully inspect volumes in wells to ensure that genomic DNA has been added. Second, results are not accepted from sample wells whose raw signals with the wild-type or mutant probe are not at least 50% of the signal obtained for no target controls. Third, we confirm homozygous mutant calls with a duplicate Invader test and a PCR-RFLP test, since R506Q homozygosity is uncommon and the clinical implications are substantial. These steps are likely to be helpful in reducing errors due to failure to add genomic DNA to a well. Fourth, since the completion of this study, we have used an expanded equivocal range of ratios of 2 to 50 to distinguish heterozygous and wild-type genotypes. In 381 additional assays performed with the expanded equivocal range after the end of this study (unpublished data), we encountered six equivocal ratios. We retyped these with Invader and PCR-RFLP assays. Four samples (ratios of 3.6, 23.4, 3.2, and 11.3) resolved as wild-type on retyping. Two samples (ratios of 5.7 and 5.9) resolved as heterozygotes on retyping. Thus, the use of an expanded equivocal range with retyping may be useful in detecting rare samples yielding ratios of 5 to 6 that are truly heterozygous.

Assays based on Invader technology provide an uncomplicated, homogeneous platform sensitive enough to allow direct detection from genomic DNA without the need for PCR amplification and specific enough to discriminate single-basepair changes. This single technology can be used to diagnose a wide spectrum of genetically based diseases,\textsuperscript{13} to detect specific single nuclear polymorphisms,\textsuperscript{12,15} to quantitate viral loads,\textsuperscript{23–25} and to test for drug resistance/mutations,\textsuperscript{26–28} providing a flexible platform with a wide breadth of applications for the clinical setting.

Acknowledgments

We thank Third Wave Technologies for supplying the reagents used to complete this study.

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