Detection of the Factor V Leiden Mutation by a Modified Photo-Cross-Linking Oligonucleotide Hybridization Assay

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**Background:** Our previously developed assay for detection of the factor V Leiden mutation (G1691A) based on a nucleic acid photo-cross-linking technology used two allele-specific capture probes and six fluorescein-modified signal-generating reporter probes. We wished to improve the sensitivity and performance of the method.

**Methods:** We developed new reporter probes with ~10-fold more fluorescein molecules than the original probes. The single, cross-linker-modified capture probe was replaced by a three-probe system, separating the probe–target cross-linking function and the allelic differentiation function. The capture probe cross-linked to either or both of two flanking probes through stem structures at the capture-probe/flanking-probe junctions. The flanking probes cross-linked to target DNA through two cross-linking sites each. Genomic DNA was extracted from 0.2 mL of whole blood, and restriction-enzyme digested to create a defined 677 bp target sequence. Preliminary genotype ranges were determined for the assay by testing of pretyped samples. We then tested 1054 clinical samples, using an automated sample processor.

**Results:** The new assay had a 10-fold increase in signal-to-background ratio. Genotype results for 1039 of 1054 clinical samples (98.6%) agreed with those of a PCR-based method. Of the 15 remaining samples, 10 produced an indeterminate result outside the defined genotype ranges, 2 yielded insufficient signal to be genotyped, and 3 gave a discordant result. All 15 samples were genotyped correctly after reextraction of genomic DNA and retesting.

**Conclusion:** The modified photo-cross-linking assay for factor V Leiden detection is a sensitive non-PCR-based assay with potential for use in high-throughput clinical laboratories.

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NAXCOR has developed a non-PCR-based nucleic acid diagnostic testing platform that is based on the use of DNA probes that are modified with photo-active cross-linking molecules (1). In a typical application, the sample of interest is first processed to render the target nucleic acids available for hybridization. Capture and reporter probes are then added and hybridized to their complementary target sequences, at which time the sample is irradiated with ultraviolet (UV) light to induce the formation of a covalent cross-link between the probes and target. The potential benefit of this approach is that by forming an irreversible probe–target hybrid, the latter steps in the assay process, such as target capture and washing, are not held to the same rigid constraints as those in a conventional hybridization-based assay. In the cross-linking assay format, both capture and washing can be performed under conditions that may denature conventional probe–target hybrids. Consequently, the cross-linking approach can provide two key benefits: increased signal as a result of more efficient target capture and decreased background as a result of more effective removal of nonhybridized DNA probes and other nonspecific signal sources.

To date, the cross-linking technology has been applied to detection of bacterial pathogens (1), quantification of viral DNA (2), assessment of gene dosage and methylation status (3), and detection of single-nucleotide polymorphisms (SNPs), specifically the factor V Leiden muta-
tion, G1691A, and the two most common mutations that are linked to hereditary hemochromatosis, G845A and C187G (4, 5). The first generation of SNP detection assays required a minimum input of 2 mL of whole blood per assay and used a sample preparation procedure that required purification of leukocytes by centrifugation after an initial erythrocyte lysis step. This was followed by boiling of the leukocyte pellet in NaOH solution to lyse the cells and to breakdown and denature the genomic DNA before hybridization. Although this procedure was straightforward, it was not easily automated and therefore not suited to the needs of a high-volume clinical laboratory.

In this report we describe the development of a modified photo-cross-linking assay for detection of the factor V Leiden mutation that incorporates improvements in cross-linking probe design and sample preparation; this modified assay is ~10-fold more sensitive than the first-generation assay. The new assay uses genomic DNA as the starting material, providing compatibility with high-throughput automated DNA extraction systems. All steps of the procedure, including reagent addition, UV irradiation, magnetic bead handling, washing, and incubations, are fully automated.

**Materials and Methods**

**PATIENT SAMPLES AND COMPARISON TESTING METHOD**

Deidentified peripheral blood samples in disodium EDTA were obtained from Stanford University Medical Center (Stanford, CA) and Quest Diagnostics Nichols Institute (San Juan Capistrano, CA). The Stanford samples were used at NAXCOR to set genotype ranges for the photo-cross-linking assay. The factor V status of these samples was determined by the PCR-based method described by Bertina et al. (6). The Quest samples used in the evaluation of the photo-cross-linking assay had previously been tested for factor V Leiden mutation status by a PCR-based assay that used the Read-ItTM detection system (Promega) (7). Once results were finalized and reported, aliquots of the original blood tubes were reextracted and tested with the photo-cross-linking assay. The blood tubes were retained by the clinical laboratory in compliance with CLIA regulations.

**GENOMIC DNA SAMPLES**

Genomic DNA samples that were derived from individuals who were wild type, heterozygous, or homozygous mutant for factor V Leiden were obtained from the Coriell Cell Repository maintained by the National Institute of General Medical Sciences. These samples were used exclusively for assay development and not as substrates for generation of controls.

**OLIGONUCLEOTIDE SYNTHESIS**

A summary of the probe designs for both the original and modified photo-cross-linking assays, including cross-linker sites and hapten modifications, is shown in Fig. 1. Three types of oligonucleotides were synthesized for the modified assay. These oligonucleotides were complementary to sequences within exon 10 and intron 10 of the factor V gene and were synthesized on an Expedite 8909 synthesizer with use of standard phosphoramidite DNA synthesis reagents (Applied Biosystems). The nucleotide numbers for the probes correspond to those in GenBank accession no. Z99572.

Allele-specific capture probes complementary to either the wild-type factor V gene or the mutant factor V (Leiden) gene. These probes contained a biotin molecule at the 3’ terminus (BioTEG CPG; Glen Research) for immobilization by streptavidin-coated magnetic beads during the assay process. Capture probe 1 was complementary to nucleotides 62922–62936 of the wild-type factor V gene. In addition to this target complementary region, the probe contained three extra thymidine residues at the 5’ end and two extra adenine residues at the 3’ end that were positioned either

![Image](312x317 to 552x476)

**Fig. 1.** Capture and reporter probe configurations for the original and modified photo-cross-linking assays for detection of the factor V Leiden mutation.

The original and modified assays use the same general design to detect and differentiate wild-type and mutant gene sequences. Each patient sample was tested with two allele-specific probe sets in separate wells. Both probe sets contain the same panel of fluorescein (Fl)-modified reporter probes, which are complementary to sequences in the factor V gene, and one of two biotinylated probes that confers allele specificity based on the presence of the complementary nucleotide for either the mutant or wild-type sequence. In both the original and modified systems, all reporter probes contain two cross-linkers for covalent attachment to the target (cross-linking is indicated by the dotted lines between the probes and target gene). In both systems, capture probes are also covalently attached to the target through cross-linking sites, although the approach differs between the two methods. Biotin molecules provide immobilization on magnetic beads during wash steps. Fluorescein is used for signal elaboration through sequential posthybridization incubation with an anti-fluorescein antibody–alkaline phosphatase conjugate and the fluorescent alkaline phosphatase substrate, AttoPhos, respectively. The original assay uses six reporter probes, each modified with two fluorescein molecules. In this embodiment, the capture probe contains a single cross-linker that reacted directly with the target. In the modified assay, each reporter probe contains ~24 fluorescein molecules (only 5 are shown) attached to the 3’ amino-modified tail. In the revised assay format, covalent binding of the capture probe to the target is mediated indirectly through cross-linking of the capture probe to each of two flanking probes that bind to contiguous sequences immediately 5’ and 3’ of the capture probe target region through stem structures created from tail sequences on each probe. The flanking probes in turn provide simultaneous attachment to the target through two cross-linkers each.
side of a coumarin-based cross-linking agent (denoted by X in the sequence). The cross-linker was derived from 7-hydroxy coumarin, 1-O-(4,4'-dimethoxytrityl)-3-O-(7-coumarinyl)-2-O-(2-cyanoethyl-N,N-diisopropyl phosphoramidite) glycerol (4): 5‘-TTT GAC AGG CGA GGA ATA AXA-3′. Capture probe 2 was synthesized in the same way but was complementary to nucleotides 62922–62937 of the mutant factor V gene sequence: 5‘-TTT GGA CAG CCA AGG GAT A AXA-3′. The bolded nucleotides indicate ???; the italicized nucleotides indicate ???.

After synthesis, the capture probes were cleaved from the solid support and deprotected by incubating the support in concentrated ammonium hydroxide overnight at 55 °C. The fully deprotected probes were purified by electrophoresis through denaturing polyacrylamide gels, followed by excision of the product bands and elution of the products (8). The purified oligonucleotides were desalted by Sep-Pak C18 treatment using the procedure recommended by the manufacturer (Waters Corp.).

Flanking probes. Cross-linker-modified probes were synthesized to the regions of the factor V gene that were immediately upstream or downstream to the capture probe hybridization sites. These probes were synthesized and purified by the same procedures as the capture probes. Flanking probe 1 was complementary to nucleotides 62890–62921 and contained three extra thymidine residues at the 5′ end. This probe was used in probe cocktails that contained either capture probe 1 (wild-type factor V sequence) or capture probe 2 (mutant factor V sequence). Flanking probe 2 was complementary to nucleotides 62937–62973 and contained two extra adenine residues at the 3′ end that were positioned either side of a cross-linker molecule. This probe was used only in the probe cocktail that contained capture probe 1. Flanking probe 3 was complementary to nucleotides 62938–62973 and contained two extra adenine residues at the 3′ end that were positioned either side of a cross-linker molecule. This probe was used only in the probe cocktail that contained capture probe 2.

Cross-linker-modified reporter probes complementary to non-allele-specific regions of exon 10 and intron 10 of the factor V gene. The procedures for the synthesis of reporter probes labeled with multiple fluorescein groups for elaboration of an enzymatic fluorescent signal have been described (3). A total of seven reporter probes were synthesized; these were complementary to nucleotides 62548–62575, 62620–62642, 62665–62697, 62705–62735, 62775–62804, 62810–62843 (exon 10), and 63096–63126 (intron 10).

Modified photo-cross-linking assay procedure

Sample preparation. DNA was extracted from 0.2 mL of whole blood by use of a QIAamp® DNA Blood BioRobot 9604® (QIAGEN) by the procedure provided by the manufacturer with one exception: the final elution volume of the DNA sample in AE Buffer [10 mmol/L Tris-HCl (pH 9.0), 0.5 mmol/L disodium EDTA] was increased from 0.20 mL to 0.23 mL. Aliquots of the extracted DNA samples (0.2 mL) were transferred to 1.5-mL microcentrifuge tubes with screw caps. Each DNA sample was digested overnight by the addition of 10 U of AluI restriction enzyme (New England Biolabs) in 25 μL of buffer [90 mmol/L Tris-HCl (pH 7.9), 8.1 mmol/L MgCl2, 90 mmol/L NaCl, 9.0 mmol/L dithiothreitol]. The next morning, 75 μL of sample diluent [15 mmol/L Tris-HCl (pH 8.0), 1.5 mmol/L disodium EDTA, 112.5 mmol/L NaOH, 0.4 g/L bovine serum albumin (BSA), 0.16 g/L calf-thymus DNA] was added to each sample. The samples were mixed by vortex-mixing, centrifuged briefly, and then heated at 95 °C for 3 min. The samples were cooled to room temperature for 5 min and then centrifuged at 12,000g for 5 min. Each processed sample was aliquoted (125 μL) into two preassigned wells of a 96-well polypropylene microplate (Greiner Bio-One, Inc.). In addition to the samples, each assay plate also contained six negative calibrators (prepared with the same reagents used for patient samples but with AE buffer that contained no DNA) and two positive controls. The positive control wells contained factor V gene DNA (10 amol) dissolved in the same solution used for the negative calibrators. The positive control DNA had been obtained previously by PCR amplification of DNA from an individual who was heterozygous for the factor V Leiden allele.

Assay setup and procedure. Determination of the factor V genotype by the photo-cross-linking assay is based ultimately on comparison of the fluorescent signals obtained from each sample after hybridization and cross-linking of the sample DNA to different sets of allele-specific probes.

The assay was carried out on an automated instrument that was designed to be used as an open system to test a variety of assays. The instrument, designed and built by Tecan Systems, consisted of storage receptacles to hold reagents, syringe pumps, and multiple pipette probes for dispensing and mixing reagents, an incubation chamber for heating samples and maintaining assay temperatures, a magnet assembly for use in washing steps, and a UV light source for the cross-linking process.

After preparation of the samples and controls, the assay reagents and microtiter plate were loaded into their appropriate holders on the instrument. On initiation of the assay protocol, the following procedures were performed: One of two different probe solutions (50 μL; containing 1.5 mol/L NaCl, 16.8 mmol/L NaOH, 170 mL/L formamide, 0.03 mmol/L phenol red, 0.06 g/L BSA) was added to the samples. The first probe solution, added to the first sample aliquot, contained the seven reporter probes (0.05 pmol each), which were complementary to a region of the factor V gene flanking the mutation site; capture probe 1 (0.5 pmol), which was complementary to the wild-type factor V gene sequence; and flanking probes 1 and 2 (1.0 pmol each). The second probe solution...
was added to the second sample aliquot and contained the same set of reporter probes as the first probe solution; capture probe 2 (0.5 pmol), which was complementary to the mutant factor V gene sequence; and flanking probes 1 and 3 (1.0 pmol each). After addition of the probe reagents to the samples and controls, 60 μL of neutralization buffer [15.6 mmol/L citric acid, 228 mmol/L NaH₂PO₄ (pH 7.0), 1.615 mol/L NaCl, 3.1 mL/L Tween-20, 272 mL/L formamide] was added to each well. The microplate was heated to 46 °C, incubated for 20 min, and then irradiated for 60 min at the same temperature. After irradiation, the plate was allowed to cool to room temperature for 10 min; 75 μg of streptavidin-coated magnetic beads (Dynabeads® M-280; Dynal) was then added to each well to capture the cross-linked probe–target hybrids via the biotin residue attached to the allele-specific capture probes. After a 25-min incubation at room temperature, the plate was washed twice by the addition of 250 μL of wash reagent 1 [1.5 mmol/L NaCl, 0.15 mmol/L sodium citrate (pH 7.0), 1 g/L sodium dodecyl sulfate] and once by the addition of 250 μL of wash reagent 2 [150 mmol/L NaCl, 15 mmol/L sodium citrate (pH 7.0), 1 mL/L Tween-20]. Immediately after the beads were washed, 100 μL of anti-fluorescein antibody–alkaline phosphatase conjugate (Dako Corp.), diluted 1:20 000 in antibody buffer [100 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 g/L Tween-20, 2.5 g/L BSA], was added to each well. The samples were incubated for 20 min at room temperature and then washed four times with 250 μL of wash reagent 2. On completion of the final wash cycle, 100 μL of an alkaline phosphatase substrate (Attophos™; Promega) was added to each well, and the plate was incubated at 37 °C for 60 min. At this time, the plate was removed from the instrument, and the fluorescent product was detected by recording the fluorescence signal with a FluoroCount™ microplate fluorometer (Perkin-Elmer Life Sciences).

DATA ANALYSIS
The net sample signal (NSS) was calculated by subtracting the mean signal of the three negative calibrators for each factor V gene probe set (wild type or mutant) from the signal obtained from a sample tested with the corresponding probe set. Negative values for the NSS were considered to be zero. Patient genotype determinations were based on the ratio of the NSS for the mutant probe set divided by the NSS for the wild-type probe set.

ASSAY PRECISION
Within- and between-run precision was assessed by testing a sample obtained from peripheral blood from a single individual. For within-run imprecision, the sample was divided into 10 aliquots, and the assay was performed 10 times on the same plate. For between-run precision, the sample was divided into 10 aliquots, and 1 aliquot was tested in each of 10 separate assay runs over a 7-day period with a common lot of reagents. All tests were performed by a single operator.

RESULTS
OVERVIEW OF THE PHOTO-CROSS-LINKING HYBRIDIZATION ASSAY FOR SNP DETECTION
The format and procedure of the original photo-cross-linking assays for detection of the factor V Leiden and hemochromatosis mutations has been described in detail elsewhere (4,5). Briefly, a sample derived from a crude leukocyte preparation was analyzed with two separate probe set combinations. The first test used a probe set containing a biotinylated capture probe complementary to the wild-type gene sequence; the second test used a probe set containing a capture probe complementary to the mutant gene sequence. Both probe sets also contained a set of reporter probes, each modified with two fluorescein molecules, that were complementary to conserved regions of the target gene surrounding the mutation site (Fig. 1).

Assay conditions were optimized for minimal hybridization and cross-linking between probe/target combinations containing a mismatch but efficient hybridization and cross-linking between fully complementary probe/target combinations. The genomic DNA from an individual with two wild-type alleles yielded a strong signal when assayed with the wild-type capture probe set and a weak signal when assayed with the mutant probe set under the assay conditions. Conversely, DNA from an individual with two mutant alleles produced a strong signal only with the mutant capture probe set. Samples that were obtained from heterozygous individuals yielded approximately the same signal with both probe sets. Determination of the sample genotype was based on the ratio of the NSS generated by the mutant probe set divided by the NSS generated by the wild-type probe set.

Typical NSS ratios observed with the factor V Leiden assay with PCR-derived target DNA and real clinical samples were 0.1, 1.0, and >10.0 for wild-type, heterozygous, and homozygous samples, respectively (4).

MODIFICATION AND IMPROVEMENT OF THE FACTOR V LEIDEN MUTATION DETECTION ASSAY
Reporter and Capture Probes. The probe cocktails in the original factor V Leiden assay used six reporter probes, each of which contained two fluorescein molecules. Enhancement of the signal-generating capability of the reporter probes was achieved by synthesizing a new set of probes that contained an increased number of fluorescein molecules in each probe. These reporter probes were synthesized by first introducing a tail containing protected amino groups at the 3’ end of each probe. The probes were then deprotected and purified by denaturing polyacrylamide gel electrophoresis. Fluorescein incorporation was achieved by reaction of each reporter probe with an amino-reactive fluorescein derivative, FLUOS (Roche Applied Science) (3). Initial assays were performed to assess the signal and background obtained from a single factor V gene reporter probe that had been modified with different length tails containing 20–50
amino groups. These results were compared with the signal and background data obtained from a reporter probe modified with two fluorescein molecules (Table 1). The results showed that the tailed probes yielded up to a 6.5-fold improvement in signal-to-background ratio compared with the original dual-fluorescein probe. On the basis of these experiments, the factor V gene reporter probes that were used in the improved assay were synthesized with 3' tails containing 40 amino groups. This tail length gave the best balance between improved signal and synthesis efficiency; probes modified with tails containing >40 amino groups produced higher signals, but synthesis yields of these longer probes were substantially lower (data not shown). A total of seven multifluorescein reporter probes were selected for use in the assay.

The coumarin-based cross-linking molecules used in these assays preferentially react with opposing thymidine residues in the complementary target strand (1). The most favored site to place the cross-linking molecule in a probe is one where it will be opposed to the central thymidine in a TTT sequence within the target. Efficient cross-linking will occur when the cross-linker is opposed to a three-base sequence containing at least two thymidine residues but becomes substantially lower if only one thymidine is present. This is not a problem in applications in which the target sequence is long because the probe sequences, and hence position of the cross-linker in the probes, can be selected so that the cross-linking efficiency is maximized. This becomes an issue, however, when designing allele-specific capture probes for SNP detection. In these cases, the probe must hybridize to a fixed target sequence surrounding the mutation site, and in addition, the probe must be short enough to differentiate between the complementary and mismatched targets.

As a result of this potential problem, a new capture probe design was developed that would remove the constraints imposed by having to directly cross-link the probe to the target. In this approach (Fig. 1), the two short allele-specific capture probes were positioned between two longer (~35 bases) flanking probes. The capture probes did not contain a cross-linking molecule in the regions of the probes that were complementary to the target DNA but were modified with a cross-linker between two adenine residues at the 3' end. In addition, the probes were also modified with a TTT sequence at the 5' end. On hybridization of a capture probe and the two flanking probes to a complementary target, two stem structures were formed, one between the AXA sequence at the 3' end of the capture probe with a TTT sequence at the 5' end of one flanking probe and the other between the TTT sequence at the 5' end of the capture probe with the AXA sequence at the 3' end of the second flanking probe. Positioning of coumarin-based cross-linkers in this type of stem structure has been shown to provide efficient cross-linking (9).

In addition to the stem regions, each flanking probe contained two additional cross-linker molecules that were placed in the target-complementary portions of the probes. Thus, UV irradiation of the sample caused the capture probe to become covalently linked to the target via cross-links that were formed between one or both of the stems at the junctions of the capture and flanking probes and by the cross-links that formed between the flanking probes and the target DNA. Experiments showed that this approach gave a 2.8-fold improvement in sensitivity over the original capture probe system and that both flanking probes contributed to this improvement (Table 2). The presence of the flanking probes had no impact on the ability of the capture probes to differentiate between the different factor V gene alleles; testing of PCR amplicons derived from wild-type, heterozygous, and homozygous mutant DNA templates yielded NSS ratios to similar those of the original assay (data not shown).

**Table 1. Effect of extent of reporter probe-fluorescein modification on signal-to-background ratio in the photo-cross-linking assay for factor V Leiden.**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Amino groups/probe</th>
<th>Fluorescein groups/probe</th>
<th>Signal-to-background ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>2</td>
<td>2</td>
<td>9.05</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>12b</td>
<td>34.6</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>18b</td>
<td>39.8</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>24b</td>
<td>48.5</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>30b</td>
<td>58.8</td>
</tr>
</tbody>
</table>

* Original reporter probe configuration; contains fluorescein molecules at 5' and 3' end of probe.
* Estimate based on mean fluorescein incorporation of 60% of the theoretical maximum.

**Sample preparation.** The original factor V Leiden assay involved a sample preparation procedure that was based on purification of leukocytes from whole blood followed by boiling of the leukocytes in NaOH solution. The boiling step was done to lyse the cells and to shear and denature the genomic DNA before hybridization. Degradation of the genomic DNA into small fragments (<1 kb) was necessary because preliminary studies showed that the efficiency of capturing the DNA target on the magnetic beads decreased as the mean size of the DNA fragments increased (data not shown). One limitation of the NaOH-based method, however, was that the DNA fragmentation was essentially random, which likely led to the formation of incomplete factor V gene target se-

**Table 2. Effect of modified capture probe configuration on signal-to-background ratio in the photo-cross-linking assay for factor V Leiden.**

<table>
<thead>
<tr>
<th>Capture probe configuration</th>
<th>Flanking probe 1</th>
<th>Flanking probe 2</th>
<th>Signal-to-background ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>−</td>
<td>−</td>
<td>30.3</td>
</tr>
<tr>
<td>Modified</td>
<td>+</td>
<td>−</td>
<td>49.2</td>
</tr>
<tr>
<td>Modified</td>
<td>−</td>
<td>+</td>
<td>61.8</td>
</tr>
<tr>
<td>Modified</td>
<td>+</td>
<td>+</td>
<td>85.7</td>
</tr>
</tbody>
</table>
quences that were only long enough to bind to a subset of the reporter probes, thus diminishing the sensitivity of the assay. To resolve this problem, we developed a sample preparation procedure that was based on automated purification of genomic DNA followed by restriction enzyme-mediated digestion of the DNA. The use of a restriction enzyme with a four-base recognition sequence, AluI, yielded genomic DNA with short (<1 kb) fragments and a defined 677-bp factor V gene target that was of sufficient length to allow the binding of sufficient reporter probes for sensitive detection of the factor V gene and determination of mutation status.

Experiments were done to show the improvement in signal-to-background ratio with the modified sample preparation procedure. Genomic DNA was either subjected to the 20-min NaOH-based boiling procedure used in the original factor V assay or was treated with AluI. The DNA samples were then processed with the modified factor V assay probe cocktail containing seven multifluorescein reporter probes and the capture/flanking probe sets. The results indicated that the restriction enzyme-based method gave a 2.1-fold increase in signal-to-background ratio.

Signal-to-background ratio of the modified factor V Leiden assay. The sensitivity of the modified assay was determined by testing genomic DNA samples that were derived from individuals who were wild type, heterozygous, or homozygous for the factor V Leiden mutation. Increasing concentrations of DNA up to a maximum of 10 μg/0.2-mL sample were processed by the restriction enzyme method described above and then analyzed in the photo-cross-linking assay (Fig. 2). As anticipated, the signal obtained from DNA derived from a heterozygous individual was split equally between the wild-type and mutant probe sets and was approximately one-half as intense as the signal obtained from DNA samples derived from wild-type and homozygous mutant individuals when tested with the wild-type and mutant probe sets, respectively. Signal was detected even at the lowest concentration of genomic DNA tested (0.5 μg/0.2 mL) for all three genotypes; however, 2.5 μg/0.2 mL was the minimum amount of DNA that yielded a fluorescence signal above the preassigned cutoff. This cutoff value, which represented a signal-to-background ratio >3 SD above the mean fluorescence values of the negative calibrators, was determined from the results generated from processing 10 full assay plates, each filled with negative calibrator. The mean + 3 SD results ranged from 1.10 to 1.21 times the mean negative calibrator values for both the wild-type and mutant probe sets. Accordingly, a signal-to-background ratio ≥1.25 times the negative calibrator values was selected for the assay cutoff.

To determine whether the sensitivity of the assay was sufficient for it to be used with DNA samples that had been extracted from whole blood by a high-throughput automated robot, we used an automated DNA extraction robot to process 172 blood samples that had been collected from patients for factor V Leiden testing. DNA was extracted from 0.2-mL aliquots of each sample and eluted in a final buffer volume of 0.23 mL. The yield of each DNA sample was then calculated by UV spectrophotometry. These analyses indicated that the mean DNA yield for the 172 samples was 7.9 μg, and only one sample yielded less than the 2.5 μg DNA required for the assay.

CLINICAL SAMPLE TESTING

Preliminary determination of factor V genotype ratios. Whole-blood samples were obtained from a routine blood chemistry testing laboratory and were used to set genotype cutoffs for the photo-cross-linking assay. The factor V status of the samples was determined at NAXCOR by a PCR-based method (6), and the samples were then processed by the photo-cross-linking assay. Samples that were determined to be heterozygous or homozygous for factor V Leiden were processed multiple times by the photo-cross-linking assay to obtain sufficient data to set the genotype ranges. In total, 890 samples were run.

Of the 890 samples, 800 had wild-type factor V gene status [mean (SD) NSS ratio, 0.18 (0.14); NSS ratio range, 0.00–0.72], 70 were heterozygous for the factor V Leiden mutation [mean (SD) NSS ratio, 1.15 (0.18); NSS ratio range, 0.751.51], and 20 were homozygous for the mutation [mean (SD) NSS ratio, 12.67 (6.6); NSS ratio range, 3.40–24.07]. We used these data to set a genotype range for wild-type samples with a lower NSS ratio limit of 0.00 and an upper limit of 0.60 (mean ± 3.0 SD). The genotype range for the heterozygous samples was set with a lower NSS ratio limit of 0.81 and an upper limit of 1.50 (mean ±
The modified photo-cross-linking assay was developed to achieve a sensitivity gain of approximately an order of magnitude over the original assay for detection of the factor V Leiden mutation. Although the overall format of the assay procedure has remained the same, the new assay contains several modifications in the areas of probe design and sample preparation.

The reporter and capture probes have been extensively revised to increase both signal-generation capability (by increased fluorescein incorporation in the reporter probes) and capture efficiency (by use of a set of capture and flanking probes) in the assay. The performance of the multifluorescein reporter probes is noteworthy because the large gain in signal over the dual-fluorescein reporter probes used in the original assay was obtained without any significant increase in nonspecific background signal.
It is possible that the extra fluorescein molecules and the long amino-spacer tails attached to these reporter probes present a structure that is less prone to nonspecific binding to the solid surfaces in the assay well (well surface and magnetic beads). Alternatively, nonspecifically bound multifluorescein reporter probes may be easier to remove during the high-stringency washing procedure that can be used with the cross-linking approach.

The capture-probe/flanking-probe system incorporated stem-based cross-linker molecules that increased the efficiency of attachment of the capture probe to its complementary target over the original system, which used capture probes that directly attached to the target. Positioning the cross-linking molecules opposite three thymidine residues in the stems ensured efficient cross-linking. Improvement was also attributable to the fact that with the capture-probe/flanking-probe approach, the capture probe needed to cross-link to only one of the two flanking probes for target capture to occur. Furthermore, by removing the cross-linkers from the target-specific portion of the capture probe, this system can be used for detection of any mutation, irrespective of whether a good cross-linking site is present near the mutation site.

Improvements were also made to the sample preparation steps before the assay. In place of the crude leukocyte preparation and NaOH-based boiling procedure used in the original assay, we developed a process that was compatible with the use of automated DNA extraction instruments. The purified DNA samples were digested with a restriction enzyme to produce a small, 677-bp target DNA fragment that was efficiently captured on the surface of the magnetic beads during the assay. Although it is possible that inhibition of the cutting process could occur in a fraction of DNA samples, our experience is that the restriction enzyme approach is highly robust and is not prone to the inhibition problems observed with other enzymes, such as the DNA polymerases used for PCR amplification.

Taken together, the modifications to the photo-cross-linking assay produced a detection limit of 1 μg of genomic DNA from factor V Leiden wild-type and homozygous mutant individuals and 2.5 μg of DNA from heterozygous individuals. These detection limits were found to be compatible with the yields obtained from the DNA extraction instrument and allowed, after preliminary assignment of genotype ratio cutoffs, further evaluation of the assay with 1054 clinical samples that had been previously tested for factor V Leiden status by a PCR-based method in a large clinical testing laboratory. To reduce the labor and complexity involved in testing the clinical samples, an automated instrument was used to perform all steps of the cross-linking assay after loading of prepared samples and controls onto the microtiter plate up to the final reading step in the fluorometer. The processing time for a full plate of 43 samples by the instrument was 4.75 h with no technician intervention necessary during this time. Because the DNA extraction and enzyme digestion steps require 2 h and 12 h, respectively, the current turnaround time for a sample is ~19 h. We note that recent development work has indicated that the digestion step can be accomplished in as little as 2 h (data not shown), which would give a turnaround time of <10 h.

Each assay plate included negative calibrators for determination of NSS ratios and positive controls that contained amplified factor V gene DNA that was derived from a factor V Leiden heterozygous individual. The experimental design for this study did not include controls derived from wild-type and homozygous mutant individuals, primarily because of space considerations. During assay development and the testing of 890 samples to set preliminary genotype ranges, we established that the modified photo-cross-linking assay showed clear allelic specificity for the three genotypes. A complete set of controls, however, will be incorporated into this assay before clinical use.

The assay also omits an internal control for independent verification of sample adequacy. The assay instead relies on satisfaction of a minimum absolute signal criterion for determining data validity. Currently the assay platform does not have the ability to generate multiplex data in a single well. Therefore, incorporation of such a control would require the use of a third well for each sample, thereby increasing the minimum DNA requirements for the assay and reducing the number of samples that can be processed on a single plate. This limitation will be addressed in future assays.

The results obtained from testing the 1054 clinical samples showed that 1039 samples (98.6%) yielded the same result as the PCR method after a single round of testing and that the three factor V alleles were clear differentiated (Fig. 3). Of the remaining 15 samples, 10 samples (0.9%) yielded NSS ratios outside the calculated genotype ranges, and 2 samples (0.2%) failed to yield enough signal to determine an NSS ratio value. These samples were reextracted and produced a satisfactory result on retesting. Because all indeterminate and invalid samples would routinely be flagged and retested in a clinical laboratory, these data indicated that there was complete agreement between the two testing methods in 1051 of 1054 samples (99.7%).

Three samples produced a discordant result between the testing methods. These samples had NSS ratios of 0.49, 0.82, and 1.22 in the initial round of testing with the photo-cross-linking assay but yielded correct results after reextraction of DNA from the primary blood samples and retesting (Table 3). To gain insight on the potential causes of these discrepant results, we performed analyses, focusing on two areas: (a) reassessing the genotype ranges based on the results of the study and (b) investigating the sample preparation step for possible causes of assay failure.

As outlined in the Results above, the genotype ratio
cutoffs for the photo-cross-linking assay were based on a preliminary analysis of a small number of samples obtained from a general blood testing laboratory. The 1054 samples used in the evaluation were a more realistic population from which to derive the assay cutoffs because these samples had been referred to the testing laboratory for factor V Leiden testing. The NSS ratios obtained from the 1054 samples (after all retests) were broken down into the three genotype groups and analyzed. The mean (SD) NSS ratio for the 910 wild-type samples was 0.15 (0.07). The mean (SD) NSS ratio for the 140 heterozygous samples was 1.17 (0.14). On the basis of these data, the genotype range for wild-type samples could be repositioned using a lower NSS ratio limit of 0.00 and an upper limit of 0.40 (mean + 3.6 SD). Likewise, the genotype range for heterozygous samples could reasonably be modified to have a lower NSS ratio limit (mean – 1.9 SD) of 0.90 and an upper limit (mean + 2.4 SD) of 1.50. The modified indeterminate zone between the wild-type and heterozygous ranges would fall between NSS ratios of 0.41 and 0.89. Because only four samples were homozygous for factor V Leiden, there were insufficient data to change the cutoffs corresponding to this genotype.

When we used these revised cutoffs, the two discordant samples with NSS ratios of 0.49 and 0.82 fell into the modified indeterminate zone between the wild-type/heterozygous ranges, leaving only a single incorrect sample (0.1%). The second major effect of these revised ranges would be to increase the number of indeterminate results from 10 (0.9%) to 19 (1.8%); however, in any assay, an increase in retest rate should be weighed against the likely benefit of increased assay accuracy. Given the relatively low retest rate associated with the modified parameters in conjunction with the consequences of erroneous results of genetic tests, we intend to adopt the revised ranges after validation in future studies.

The remaining discordant sample was homozygous for the factor V Leiden mutation but produced an NSS ratio (1.22) that was consistent with heterozygosity in the photo-cross-linking assay. Analysis of the raw data set of the assay plate that contained this sample showed that the plate also produced 3 of the 10 indeterminate samples and 1 of the 2 invalid samples obtained in the total evaluation (25 assay plates were run in total). On further review of the evaluation data, we noted that a second assay plate that contained a disproportionately higher number of indeterminate samples (2 of 10) contained several samples that were yellow in color. This plate also contained one of the discordant samples (NSS ratio, 0.82) that would be reclassified as an indeterminate result by the revised genotype ranges discussed above. We believe that the increased number of indeterminate, invalid, and discordant samples on these plates stemmed from a problem encountered during the DNA extraction procedure because retesting of these samples led to correct genotype assignments.

Subsequent experiments with the reagents used for the extraction process have shown that yellow samples can be generated if the initial protease reagent is omitted from the procedure. Testing of these incorrectly processed samples in the photo-cross-linking assay yielded an increased frequency of indeterminate and incorrect results (data not shown). We have also observed the intermittent failure of the DNA extraction robot to add the protease to the samples. We believe that this occurs if the racks of disposable pipette tips are not seated correctly in their receptacles on the instrument or if the dispenser head holding the disposable tip adapters is not aligned correctly. Either of these problems would lead to poor fitting of the tips and inefficient aspiration and delivery of the extraction reagents. It is possible, therefore, that this problem led to at least some of the indeterminate, invalid, and discordant sample results in the study. We are continuing to investigate the cause of these sample preparation-induced effects.

There are several methodologies available for the determination of factor V Leiden status. Most of these procedures require amplification of the factor V gene by PCR and a detection step that is performed either separately, after amplification, or in real time during amplification (10,11). The PCR-based method used on the samples in this study used the Read-It detection system (Promega) (7). A PCR-independent technology, however, offers some distinct advantages over a PCR method. For example, the assay does not suffer from problems inherent to PCR, such as false results attributable to reaction contamination. In addition, there are potential cost savings; direct methods such as the photo-cross-linking assay do not require the use of separate rooms, which are necessary to prevent amplicon contamination, and the assays themselves simpler than PCR-based assays and do not require highly trained personnel.

One exception to PCR-based factor V Leiden assays is the Invader® system developed by Third Wave Technologies, Inc. (12). The Invader assay is based on the use of Cleavase® enzymes, which recognize and cleave structures that form when two oligonucleotides (an Invader probe and a probe complementary to either the wild-type or mutant factor V gene sequence) overlap each other when hybridized to a complementary target. Because the cleavage reaction will not occur with mismatched probe–target complexes, this enzyme-based technology should be able to differentiate wild-type and mutant sequences. The Invader assay requires ~10-fold less input DNA than the modified photo-cross-linking assay and offers sample turnaround times within 1 working day (12).

In one clinical sample study (12), the performance of the Invader assay for factor V Leiden appeared to be similar to that of the photo-cross-linking assay in terms of overall percentage of indeterminate and invalid samples that required retesting (1.7% for the Invader assay and 1.1% for the photo-cross-linking assay). In the same study, the Invader assay incorrectly genotyped 4 of 1369 samples (0.3%); however, the results of a second study indicated
that the Invader assay misread a substantially higher percentage of samples, 2.2% (17 of 758) (13). Only 3 of 1054 (0.3%) samples were incorrectly genotyped by the photo-cross-linking assay in this study (0.1% after reassignment of the assay cutoffs); however, it should be stressed that a true comparison of these two non-PCR assays would require side-by-side evaluations with the same sets of clinical samples. An important question for any mutation detection technology concerns the level of accuracy that it must achieve for it to be acceptable for use in a clinical diagnostic laboratory. Studies have demonstrated that there are platforms capable of error-free genotyping of 31 mutations in the cystic fibrosis gene in a 1000-sample comparison (14). The current factor V Leiden testing platform used at Quest Diagnostics has demonstrated error-free genotyping in three 1000-sample comparisons, including the current study (unpublished results). We therefore feel that before the introduction of a molecular genotyping assay into a high-throughput laboratory, a platform should demonstrate error-free allele assignment in at least 1000 clinical samples. This initial comparison revealed three undetected discrepant allele assignments. Two of these were resolved after reassignment of thresholds and the third was likely attributable to a sample preparation-related problem; however, further refinement of the photo-cross-linking technology and a new error-free 1000-sample comparison study will be needed to ensure that this technology can be confidently introduced into a high-throughput clinical laboratory.

In conclusion, the modified photo-cross-linking direct assay for detection of the factor V Leiden mutation is a sensitive and automated non-PCR-based assay. We are currently further optimizing the performance of this technology as well as expanding the number of SNP detection assays that can be performed on the automated platform to meet the needs of high-throughput clinical laboratories.

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