Evolving Methods for Single Nucleotide Polymorphism Detection: Factor V Leiden Mutation Detection

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Background: The many techniques used to diagnose the Factor V Leiden (FVL) mutation, the most common hereditary hypercoagulation disorder in Eurasians, and the most frequently requested genetic test reflect the evolving strategies in protein and DNA diagnosis. **Methods:** Here, molecular methods to diagnose the FVL mutation are discussed. **Results:** Protein-based detection assays include the conventional functional activated protein C resistance coagulation test and the recently reported antibody-mediated sensor detection; and DNA-based assays include approaches that use electrophoretic fractionation e.g., restriction fragment length polymorphism, denaturing gradient gel electrophoresis, and single-stranded conformational PCR analysis, DNA hybridization (e.g., microarrays),

DNA polymerase-based assays, e.g., extension reactions, fluorescence polarization template-directed dye-terminator incorporation, PCR assays (e.g., amplification-refractory mutation system, melting curve analysis using real-time quantitative PCR, and helicase-dependent amplification), DNA sequencing (e.g., direct sequencing, pyrosequencing), cleavase-based Invader assay and ligase-based assays (e.g., oligonucleotide ligation assay and ligase-mediated rolling circle amplification). **Conclusion:** The method chosen by a laboratory to diagnose FVL not only depends on the available technical expertise and equipment, but also the type, variety, and extent of other genetic disorders being diagnosed. J. Clin. Lab. Anal. 25:259–288, 2011.

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Key words: DNA assay; Factor V Leiden; coagulation tests; ELISA; immuno-optical sensor; restriction fragment length polymorphism; denaturing gradient gel electrophoresis; single-strand conformation polymorphism; microarrays; gold particle probes; single-base extension; fluorescence polarization template-directed dye-terminator incorporation; amplification–refractory mutation system; melting curve analysis using real-time quantitative PCR; helicase-dependent amplification; sequencing; invader; oligonucleotide ligation assay; ligase-mediated rolling circle amplification

INTRODUCTION

One of the most frequently performed clinical genetic tests is for the Factor V Leiden (FVL) mutation (1,2). This mutation, first described in 1994, is the most common hereditary hypercoagulability disorder amongst Eurasians. The FVL disorder is an autosomal dominant condition that displays incomplete dominance. The prevalence of the mutation varies between 1 and 13%, depending on geographic site, ethnicity, and variable sensitivity of the detection assays (3). Homozygotes and heterozygotes for the FVL mutation have an ~80- and ~8-fold increased risk of developing venous thrombosis, respectively (4,5). Deep vein thrombosis in the heart or lung may cause pulmonary embolisms or transient ischemic attacks (mini-strokes) (6).

The FVL mutation in combination with other clotting risk factors, including smoking, hormonal contraception,

Abbreviations: APC, Activated protein C; ARMS, Amplification Refractory Mutation System; ASO, Allele-specific oligonucleotide; Cp, Crossing point; Ct, Cycle threshold; ddNTP, 2',3'-dideoxynucleotide triphosphate; DGGE, Denaturing Gradient Gel Electrophoresis; dNTP, Deoxynucleotide triphosphate; ELISA, Enzyme Linked Immunosorbent Assay; FDA, Food and Drug Administration; FP-TDI, Fluorescence Polarization Template-directed Dye-Terminator Incorporation; FRET, Fluorescence Resonance Energy Transfer; HAD, Helicase Dependent Amplification; HRP, Horseradish peroxidase; L-RCA, Ligase-mediated Rolling Circle Amplification; LSO, Locus-specific oligonucleotide; MTHFR, Methylene-tetrahydrofolate reductase; OCP, Open circular probes; OLA, Oligonucleotide Ligation Assay; Q-PCR, Quantitative PCR; RFLP, Restriction Fragment Length Polymorphism; SAM, Self-assembled monolayer; SAV, Streptavidin; SNP, Single Nucleotide Polymorphism; SSCP, Single Stranded Conformational PCR; T_m , Melting temperature; TMB, 3,3',5,5'-tetramethylbenzidine; WT, Wild type.

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estrogen hormone replacement, and recent surgery, further increases the risk for venous thromboembolism and stroke (7,8). Women with a FVL mutation have an increased risk of miscarriage and stillbirth owing to clot formation in the umbilical cord, placenta, or fetus (9). Coagulopathies leading to a hypercoagulable state have been associated with retinal artery occlusion, a common vision threatening disease (10).

The high prevalence and medical risk associated with the FVL mutation create a need for effective and accurate detection methods. Confirmed presence of the FVL mutation allows design of appropriate patient management protocols and identification of individuals and families at risk of future venous thromboembolism. Currently, several technologies are used for detection of FVL. This article will describe the principles behind the standard and newly developed DNA tests, including our own using ligase-mediated rolling circle amplification (L-RCA). As the field of DNA sequencing is changing rapidly, this unit represents a snapshot of this particular moment. Table 1 summarizes characteristic of each of the discussed method (Table 1).

FACTOR V AND THE COAGULATION CASCADE

The Factor V (FV) protein has an important role in the coagulation cascade where an FV is "activated" to FVa through cleavage by thrombin at three sites. The FVa in a complex with activated Factor Xa protease converts prothrombin (Factor II) to thrombin (Figs. 1 and 2; for recent review, see Owens et al. (11)). Thrombin cleaves fibrinogen to produce a dense network of fibrin, which makes up the majority of a blood clot.

The FV gene is located on the chromosome 1q23, spans 70 kb, and consists of 25 exons with several types of internal repeats and organized in domain structures: A1-A2-B-A3-C1-C2 (Fig. 1). The FV protein is a glycoprotein and the primary sequence is 40% identical to coagulation Factor VIII. Both FV and Factor VIII are ~30% identical to the A-domain in the human multicopper oxidase, ceruloplasmin (12).

The FV protein is composed of a heavy chain (110 kD) and a light chain (78 kD). The heavy chains contain domains A1 and A2 and the light chains contain domains A3, C1, and C2. The B domains contain all three of the thrombin cleavage sites. The inactive form of FV circulates with little/no procoagulant activity. Before its participation in the coagulation cascade, FV is activated through limited proteolysis by thrombin of three peptide bonds at Arg709, Arg1018, and Arg1545, which releases the B domain, whereas the heavy and light chains remain noncovalently held together by calcium ions (13). Activation of FV exposes binding sites in the molecule for prothrombin (Arg697–Arg709) (14) and Factor X protein (Arg501, Arg510, Ala511, Asp513, Asp577, and Asp578) (15–17).

Tissue injury or inflammation initiates the coagulation cascade through inflammatory mediators that induce the expression of Tissue Factor protein in blood vessel endothelial cells (Fig. 2). Then, Tissue Factor protein binds circulating Factor VII, and an autocleavage reaction produces a Tissue Factor-activated Factor VII complex (Tissue Factor–Factor VIIa). This complex catalyzes the conversion of Factors IX and X to their activated forms (Factors IXa and Xa, respectively). Alternatively, Factor X may be activated by tenase, a Factor VIIIa–Factor IXa complex. Then, prothrombinase, a complex of Factor Xa with FVa, cleaves prothrombin to thrombin.

The three anticoagulant mechanisms involve serine proteases. The antithrombin pathway involves the removal of a circulating thrombin–antithrombin complex by liver antithrombin, preventing Factor X activation. In the Tissue Factor inhibitor pathway, Tissue Factor synthesized by endothelial cells in response to injury inactivates the Tissue Factor–Factor VIIa complex in the presence of Factor Xa. In the third pathway, activated protein C (APC) inactivates Factors Va and VIIIa, thereby blocking thrombin generation. APC inactivates FV by cleavage at arginine residues at positions 306, 506, and 679, within both heavy and light chains, destroying the prothrombin and Factor X-binding sites (16).

The FVL mutation is a single nucleotide polymorphism (SNP), a G1691A change, within exon 10 that results in an arginine to glutamine substitution at amino acid position 506 (R506Q) that decreases APC affinity for FV, thereby decreasing cleavage. The inhibition of APC degradation leads to excess thrombin in blood and increased risk of blood clotting. APC resistance is regarded as the most prevalent coagulation abnormality associated with venous thrombosis (18). The FVL mutation is responsible for 90–95% of APC resistant cases (19).

PROTEIN DETECTION

Functional Coagulation Tests

Initially, and even today, functional testing of blood coagulation time is used to determine coagulation deficiencies (20). In this assay, APC is used to degrade Russell viper venom activated FV (FVa) in plasma test samples (Fig. 3). Leiden mutation containing FVa molecules are APC resistant (see above). In the detection phase, the FV-dependent prothrombin activator, Noscarnin, is added to induce clotting and the clotting time is recorded. When activated, FV molecules in the samples are eliminated, the velocity of prothrombin activation is slow, and the clotting time is long. When the FVa elimination is incomplete (e.g., owing to a FV Leiden mutation), the velocity of prothrombin activation is high and the clotting time is short. Various APC resistance assays are available, with

TABLE 1.

Method	Principle	Format	Advantages	Disadvantages
Protein detection				
Functional coagulation tests	Observation of clotting time after APC addition	Electromechanical or photo-optical clot detection	Quick Inexpensive Minimum labor Many could be done with minimal skill	Confounding factors can interfere with results Does not verify presence of FVL mutation
Immuno-optical biosensor	FV WT or FVL antibody on sensor	Fiber-optic chamber Fluorometer	Quantitative	Lack of specific antibodies
Electrophoretic fractionation				
Restriction fragment length polymorphism (RFLP)	Digestion with endonuclease recognizing SNP site	Thermocycler Polyacrylamide or agarose gel	Inexpensive	Several manual steps Handling of potentially hazardous gels
Denaturing gradient gel electrophoresis (DGGE)	Mobility of amplicon on denaturing gradient	Thermocycler Polyacrylamide gel with denaturing urea/formamide gradient	Distinguishes between rare mutations	Stringent experimental conditions Hard to interpret and standardize results Handling of potentially hazardous gels
Single-strand conformation polymorphism (SSCP)	Mobility of amplicon in nondenaturing gels	Thermocycler Polyacrylamide gel	Distinguishes between rare mutations	Stringent experimental conditions Hard to interpret and standardize results Data analysis cannot be automated Handling of potentially hazardous gels
DNA hybridization				
Microarrays using gold nanoparticles	Differentially labeled allele-specific hybridization probes	DNA chip reader Microarray scanner	No preassay PCR required High throughput Can be automated	Equipment expensive Not time or cost effective if few samples or few SNPs
Electronic microarray hybridization	Differentially labeled allele-specific hybridization probes	DNA chip reader Microarray scanner	High throughput Automated data acquisition	Expensive equipment Not time or cost effective when few samples or few SNPs analyzed
Bead Arrays	Extension ligation of differentially labeled allele-specific hybridization probes	Thermocycler BeadExpress reader	No preassay PCR required High throughput Automated data acquisition	Equipment expensive Not time or cost effective when few samples or few SNPs analyzed
DNA polymerase assays				
<i>Extension reactions</i>				
Single-base extension ELISA	Two reactions using allele-specific primers and biotinylated allele-specific dNTPs	Thermocycler ELISA reader	Inexpensive	Many labor steps
Allele-specific primer extension	Allele specific primer extension using fluorescently labeled dNTPs hybridized to microarray	DNA chip reader Microarray scanner	High throughput Automated	Training required to operate instrument and perform software analysis Not time or cost effective if few samples or few SNPs

TABLE 1. Continued

Method	Principle	Format	Advantages	Disadvantages
Fluorescence polarization template-directed dye terminator incorporation	Single base extension with differentially labeled allele-specific dideoxy dye terminators	Thermocycler FP reader	Inexpensive Many could be done with minimal skill	Many labor steps
PCR assays Amplification refractory mutation system (ARMS)	Allele selective amplification, primers with mismatched 3'-residue(s) do not extend	Thermocycler Agarose gel	No manipulation of PCR product	Handling of potentially hazardous gels
Melting curve analysis using FRET Q-PCR	Allele-specific acceptor and donor probes bind in close proximity to target sequence	Real-time PCR thermocycler	Quick Automated data acquisition Allows simultaneous detection of two SNPs High throughput	Training required for instrument and analysis
Helicase-dependent amplification	Isothermal amplification with allele-specific, differentially labeled primers, and site-specific capturing	Thermocycler or waterbath BEST cassette	Isothermal reaction Minimum labor or knowledge required	Low throughput
DNA sequencing Dideoxy sequencing	Fluorescence detection of DNA synthesis during step-wise addition of differentially labeled chain-terminating ddNTPs	Sanger sequencer	Gold standard method Can get confirmation from other strand	Heterozygotes can be missed Multiplexing limited
Pyrosequencing	Detection of released pyrophosphate from DNA synthesis during step-wise addition of dNTPs	Pyrosequencing instrument	High throughput	Expensive equipment Multiplexing limited
Cleavase Invader assay	Invader, allele-specific probe and target forms three partite structure that is recognized and cut by cleavase enzyme	Thermocycler FP meter	Can be a completely PCR-free method	Large amounts of DNA needed Requires specific probes and FRET cassette that is only available from the company (Hologic Inc.)
Ligase Oligonucleotide ligation assay (OLA)	A fluorescently labeled common probe and biotinylated allele-specific probe are hybridized and ligated on target in case of match	Thermocycler ELISA reader	Inexpensive Can be automated	Stringent experimental conditions
Ligase-mediated rolling circle amplification	A synthetic probe is ligated in the presence of target SNP forming a circular template amplified with RCA	Real-time PCR thermocycler	Can be automated	Several manual labor steps Stringent experimental conditions

Fig. 1. Structure of Factor V. (A) Organization of gene: the size scale in kilobases (kb) is shown at the top of the figure. The second line is schematic representation of the human Factor V gene drawn to scale with exons (rectangles) connected by lines representing the introns. The number for each exon is indicated. Figure modified from Cripe et al. (84). (B) Domains in the Factor V protein. The thrombin cleavage sites indicated by arrows occur on the carboxy side of Arg-709, Arg-1018, and Arg-1545 amino acid residues. The identity of the domains is indicated by the letters inside the boxes. The regions corresponding to the heavy chain, connecting region, and light chain of factors are indicated at the bottom.

Fig. 2. Coagulation cascade. Damage to endothelial walls induces expression of Tissue Factor protein that binds to Factor VII, setting off a cascade involving sequential serine protease cleavage activations (indicated by pointed arrows): Tissue Factor protein–Factor VIIa complex converts FX to FXa. FX can be activated by Factor IXa and its cofactor Factor VIIIa. FXa along with its cofactor Factor Va converts prothrombin to thrombin, which converts fibrinogen to fibrin. Proteins that inhibit coagulation are indicated in boxes. Pointed arrows, activation; blunt arrows, inhibition. Figure taken from Marshall (85).

false negatives and false positives for FVL reported near 1% (21). Conditions owing to lupus disease or to anticoagulant or warfarin and heparin anticoagulant therapies as well as mutations, other than the Leiden polymorphism, can interfere with clotting. For instance, novel FV mutations can lead to inactivation by APC (22). Thus, clotting

tests can reveal a general increased risk of clotting, but does not prove the presence of the FVL mutation. Diagnostica Stago (Asnieres, France; <http://www.stago.com>), American Diagnostica (Stamford, CT; www.american Diagnostica.com), DiaPharma (West Chester, OH; <http://www.diapharma.com>), and Centerchem, Inc.

Fig. 3. Coagulation Test, Activated Protein C (APC) resistance assay. Snake venom FV activator converts Factor V in patient's plasma to activated FV (FVa). Then, FVa is eliminated by the addition of APC, and FVa-dependent prothrombin activator, Noscarin, is added and the time taken for blood clotting to occur, and the partial thromboplastin time (aPTT) is measured. Two aPTT reactions are performed, one with APC and one without APC. The results are expressed as the ratio of the values obtained with the two aPTT tests: $\text{aPTT} + \text{APC (seconds)} / \text{aPTT (seconds)} = \text{APC resistance ratio}$. An aPTT ratio below two (cutoff) indicates APC resistance.

(Norwalk, CT; <http://www.centerchem.com/>) are a few of the many companies marketing Food and Drug Administration (FDA)-approved kits (23).

Enzyme-Linked Immunoassay

A major advantage of enzyme-linked immunoassay (ELISA)-based SNP detection is that many clinical laboratories have the needed instrumentation. The major disadvantages include the difficulty of detecting SNPs that change a single amino acid residue. Only one ELISA test has been described, as in the development for FVL protein mutation detection, i.e., the immuno-optical sensor assay.

Immuno-optical sensor

This system determines the amounts of FV or FVL molecules using antibodies in a sandwich immuno-reaction on an optical quartz fiber (24). This test was developed with monoclonal antibodies against FV, and against FVL generated by injecting into mice a 20 amino acid sequence (conjugated with a carrier protein to increase the immunogenicity) around the mutation region, then screening isolated hybridoma cell lines for antibody. Screening was done using ELISA tests and the injected 20-mer amino acid sequences to identify for high affinity and selectivity antibody.

DNA ASSAYS

DNA assays are usually easier and more accurate than phenotyping, and stored DNA can be used for retrospective studies. Most importantly, only a DNA test can establish whether a patient has the FVL mutation. Below, we describe various DNA tests that are in clinical use or in development. The tests are grouped on the applied technology.

Electrophoretic Fractionation

Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) was the first technique used to detect DNA polymorphisms and serves as the standard for judging other DNA assays. This method detects both insertions and deletions and restriction site polymorphism. Most RFLPs are owing to restriction site polymorphism, as in the FVL test that allows or prevents cleavage producing different length fragments (i.e., RFLPs) distinguished electrophoretically.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) takes advantage of the melting temperature differences of double-stranded DNA fragments with even a single SNP difference (25). In DGGE, a GC-clamp (a 30–60

nucleotides GC rich sequence) is added to the amplicon. Then, the recombinant molecule is fractionated by electrophoresis in the presence of a denaturing gradient, created using a mixture of urea and formamide or through temperature regulation. The GC-clamp sequence remains double stranded during electrophoresis, whereas the melting temperature (T_m) of the target DNA is sequence dependent. Above the T_m , formation of a Y structure prevents further movement in the polyacrylamide gel. DGGE for FVL was developed by Chan et al. (26) and Patri et al. (27).

Single-stranded conformational PCR analysis

Single-stranded conformational PCR (SSCP) analysis is based on electrophoretic mobility differences of single-stranded DNA, reflecting conformational changes ("conformers") imposed by even a single SNP (28). The conformers are created by heating (melting), and then rapidly cooling DNA to promote intramolecular folded molecules. The conformers are fractionated under nondenaturing electrophoretic conditions using polyacrylamide. The assay was applied to FVL detection in 1996 (29,30). In practice, both SSCP and DGGE are difficult to perform and have low throughput. High-resolution melting curve analysis is a preferred mutation detection based on DNA conformation, because it has higher efficiency and is less time-consuming than conformation sensitive gel electrophoresis.

DNA Hybridization

Microarrays

DNA chips are composed of microarrays of immobilized nucleic acid on a solid support, in some cases utilizing multiple wells and hybridization of the complementary sequence (31). In most implementations, denatured single-stranded genomic DNA is hybridized to an immobilized capture oligonucleotide probe. In other protocols, multiple genomic DNA samples are immobilized and then analyzed by hybridization to a gene probe.

Microarrays using gold particle probes

The Verigene System (Nanosphere Inc., Northbrook, IL; <http://www.nanosphere.us>) is a microarray-based platform that allows multiplex SNP genotyping, including FV, in human genomic DNA without the need for target amplification (Fig. 4A). In this assay, allele-specific amine-modified capture probes spanning the SNP site are attached to specific sites on a glass surface. Heat-denatured ultrasonicated genomic DNA is hybridized to the immobilized capture probe. Signal is generated by deposition of silver around a gold nanoparticle, covalently bound to a signal oligonucleotide that

hybridizes to a sequence neighboring the genomic SNP site (Fig. 4B). Alleles are distinguished because they hybridize to spatially separated capture probes. Verigene evaluated 176 samples and found the specificity for FVL was 98.3% as compared with DNA sequencing results (32). Individual accuracies for the FV, prothrombin and MTHFR genotypes were 98.3, 94.9, and 92.6%, respectively. The accuracy for all three tests combined was 95.3% and the three-call rate (percent of samples for which calls were made for all three alleles) was 90.9%. Sequencing results confirmed that all genotypes called by the Verigene test were correct. The tested samples were 162 homozygous WT and 14 heterozygous for FVL; 171 homozygous WT and 5 heterozygous for prothrombin G20210A; and 80 homozygous WT, 81 heterozygous and 15 homozygous for MTHFR. This enzyme-free technique eliminates the need for both target amplification and genomic DNA complexity reduction.

Electronic microarray hybridization

An electronic microarray platform, the NanoChip system by Nanogen (San Diego, CA; <http://www.epochbio.com>) for FVL was developed in a multiplex format that also detects the prothrombin G20210A mutation for research use only. This system is no longer available. The NanoChip device had an array of 100 pads, synthetic polymer hydrogen, on silicon, each connected to an electrode (Fig. 5A). Each pad was electronically loaded with a specific biotinylated PCR amplicon. The biotinylated molecules were bound by streptavidin within the hydrogel. After the double-stranded amplicons were denatured with NaOH and washed with a high-salt buffer, allele-specific fluorescently labeled reporter probes were concentrated in the hydrogel pads for hybridization. At a predetermined discrimination temperature (32°C for FV), mismatched probes were removed while matched probes remained. Fluorescent signal was obtained from the remaining reporter probes hybridized to the target DNA (Fig. 5B).

The NanoChip system was compared with melting curve analysis on 225 samples, and the results of the methods showed 100% agreement for both the FVL and the prothrombin G20210A mutation, when compared with results obtained with melting curve analysis (33). In another study, when compared with melting curve analysis and RFLP, 789 of 800 individual amplicons analyzed on the NanoChip were in complete agreement with both other assays. Eleven tests were "no calls" (uninterpreted by the NanoChip system) resulting from failed pre-PCR reactions (34). In another study, 635 samples previously characterized by Third Wave Technologies' Invader assay was tested with the NanoChip system and 17 samples were discordant with the

Fig. 4. Microarray. (A) Primary hybridization of 5'-end of target DNA to capture probes with the SNP at the 3'-base, followed by a secondary hybridization of the LSO 3'-end to gold nanoparticle-labeled probes. Visualization is achieved by staining with silver reagent. (B) Example of a Verigene F5/F2/MTHFR microarray. Signals are derived from deposition of silver around gold nanoparticles that localize to features on a fixed microarray surface. Three 2 × 6 grouping features contain duplicates of FV, FII, and MTHFR genotyping assays in alternating order. The image shown was automatically scanned and analyzed by a Verigene Reader to report results of homozygous WT for FII and heterozygous for both FV and MTHFR variants. Three grouping features contain duplicates of each genotyping assays in alternating order. Figure taken from Lefferts et al. (32).

Fig. 5. Electronic microarray. (A) Biotinylated PCR amplicons, bound by streptavidin (SAV) to specific array sites on a nanochip, are hybridized with allele-specific fluorescent reporter probes (the FV WT probe and the FVL probe are labeled with Cy3 and Cy5, respectively). After excess probes are removed, emission from remaining bound reporter probes is measured and genotypes determined from the relative level of hybridization. (B) Shown are between-cartridge variation of fluorescence ratios obtained over eight runs using seven cartridges as scatter plots of Cy5 (FVL) and Cy3 (FV WT) fluorescence ratios for heterozygous samples genotyped in the NanoChip Molecular Biology Workstation. The means are shown by the solid lines. The dashed lines indicate two standard deviations from the mean ratio. Figure modified from Erali et al. (33).

Invader result (35). Sequencing confirmed that the NanoChip system was 100% accurate in characterizing FV WT, heterozygote, and homozygote samples compared with 99.2, 90.2, and 100%, respectively, for the Invader assay.

The electronic microarray approach is faster than RFLP, and allows automation. Furthermore, the ability to produce reconfigurable electric fields allows used reporter probes to be stripped from the pads and samples to be analyzed with additional probes.

Electronic targeting or oligonucleotide addressing and hybridization to array pads allow selective testing, significantly decrease DNA hybridization time because the target is concentrated at the test sites, and allow the use of electronic stringency to improve hybridization specificity (36).

Bead arrays

Bead Array technology is based on the random self-assembly of a bead pool, i.e., self-assembled monolayer (SAM), as opposed to conventional microarrays that are preformed by spotting or synthesizing probes onto a surface (37). Recently, Illumina received FDA clearance for an extension ligation-based microbead assay for FVL typing, named the GoldenGate Genotyping Assay on a VeraCode/BeadXpress platform.

In this assay (Fig. 6A), genomic DNA is biotinylated through a heat-activated chemical reaction, immobilized to streptavidin-coated paramagnetic particles, and simultaneously annealed to two allele-specific oligonucleotides (ASOs) for each SNP and one locus-specific oligonucleotide (LSO). Each of the three oligonucleotides contains a sequence complementary to genomic DNA and a “universal” PCR primer site. In addition, the LSO also contains a unique address sequence that targets a particular bead type.

Annealing of the primers to the immobilized genomic DNA is carried out by ramping the temperature from 70 to 30°C over a 2 hr period. At 30°C, a master mix for extension and ligation is added to extend perfectly matched ASO(s) to fill the gap between the ASO and the LSO primers. Then, the nick is sealed to form double-stranded DNA templates that are amplified with three primers, two allele-specific differentially labeled with Cy3 and Cy5, and one common primer. The amplification product is hybridized to a bead through its address sequence. The SAM is washed and then imaged using a Bead Array reader. A scan of a 96 element array in a SAM takes approximately 90 min.

Proprietary OligoDesigner software is used to design the GoldenGate genotyping assays. This software evaluates sequences flanking the targeted SNP, such as degree of homology to repeated sequences across the genome, palindromic sequences, GC and AT content, and neighboring polymorphisms. The T_m of the allele-specific sequence is designed to be 60°C (57–62°C), whereas the locus-specific probe is designed to be 57°C (54–60°C).

A high degree of miniaturization can be achieved by exploiting the intrinsic size of the beads and patterned substrate (38). The primary advantage of this approach is dense packing with wide scalability. Curiously, the assay seemed to have dye intensity biases between the

two channels used in fluorescence detection that was not eliminated after the data was processed with BeadStudio software (39).

DNA Polymerase Assays

Extension reactions

Single-base extension ELISA assay

This assay was developed for the detection of FV Leiden by Wilde et al. (40). First, a locus-specific preassay PCR is performed, then two separate allele-specific single-base primer extension reactions are done (Fig. 7A). In the extension reaction, a 5'-fluorescein (F)-labeled primer is annealed to the PCR amplicon adjacent to the SNP site, and a single biotinylated dNTPs specific for one of the alleles added to the reaction. The reactions are transferred to a streptavidin-covered well where the biotin-labeled products are bound and unbound primers washed away. Anti-F-conjugated horseradish peroxidase is added and a color change in TMB (3,3',5,5'-tetramethylbenzidine) substrate indicates the presence of the enzyme (Fig. 7B).

Pronto Diagnostics (Rehovot, Israel; <http://www.prontodiagnostics.com>) manufactures kit based on this assay for detection of the FVL mutation, prothrombin G20210A mutation, and methylenetetrahydrofolate reductase (MTHFR C677T). The results of testing 284 patient samples gave identical results with RFLP analysis: 0% false positive for all three mutations and 0, 0.84, and 0.58% false negatives for FVL, prothrombin G20210A, and MTHFR C677T, respectively (41).

Allele-specific primer extension

In May 2007, AutoGenomics Inc. (Carlsbad, CA; <http://www.autogenomics.com>) received clearance from the FDA to market the INFINITI System Assay for the detection of FVL, using real-time PCR with fluorescence resonance energy transfer (FRET) probes. The Infiniti analyzer is a fully automated microarray platform. The assay is based on allele-specific primer extension (Fig. 8). The 3'-end of the SNP extension primer contains nucleotide complementary to a target SNP. Allele-specific primer extension occurs when there is a match using a fluorescently labeled deoxynucleotide triphosphate for several extension cycling steps. The automated analysis is done on BioFilmChip that has an array of immobilized capture probes, each with a unique sequence, “zipcode,” not found in the human genome. Specific zipcodes are immobilized at each predetermined spot to the microarray. The 5'-end of the allele-specific primer (“anti-zipcode”) hybridizes to immobilized capture probe sequences. Fluorescence at a specific array element is observed when the

Fig. 6. Bead Arrays. (A) Biotinylated target DNA is immobilized to SAV-coated beads. The two allele-specific oligos (ASOs) distinguish between the SNPs. A locus-specific oligo (LSO) binds downstream of the SNP of the PCR product, has a primer site P3 and an address sequence complementary to sequence attached to a second bead. Following allele-specific primer extension and ligation reactions, a set of fluorescently labeled allele-specific primers (Cy3 and Cy5 labeled) is added for PCR. (B) The address sequence hybridizes to its cognate sequence on a second bead and the fluorescence on each bead is quantified, resulting in a signal associated with a particular address sequence and the presence of Cy3, Cy5, or both signals on a given bead type indicates FV WT, FVL, or FV WT/FVL genotypes. Figure modified from Lin et al. (86).

allele-specific primer has been extended (Fig. 8). One hundred seventy-five samples from three clinical sites were analyzed with the INFINITI System Assay for

Factor II and FV to a FDA-cleared device (not specified). Identical results were obtained for 89.4% for 113 FV WT samples, 100% for 47 heterozygotes,

and 95.7% for 15 FVL homozygotes. On a repeated run, 100% of all genotypes were identical (AutoGenomics Inc., Carlsbad, CA).

Fluorescence polarization (FP), first described by Perrin in 1926, detects change in molecular size, and has been used for decades in clinical assays to detect protein interactions, e.g., antibody–antigen binding (42). In FP, polarized monochromatic light irradiates a fluorophore in solution. When the fluorophore remains stationary between excitation and emission, polarized fluorescent light is emitted into the same plane as the

FP value is expressed as the ratio of fluorescence detected in the vertical and horizontal axes and, therefore, is independent of the fluorescence intensity, an advantage over other fluorescence detection.

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Fig. 8. Allele-specific primer extension. A multiplex PCR is followed by allele-specific primer extension. Hybridization and the extended primers, incorporating Cy5-labeled dCTPs, are hybridized to chips using the AutoGenomics ZipCode anti-ZipCode technology and fluorescence at each set of the SNP-specific sites is read by a confocal microscope. Only the cytosines (C) in the sequences are shown to illustrate fluorescently labeled dNTPs. The fluorescent results are genotyped by automated analysis.

nucleotide, labeled with a fluorophore (low FP value), into a primer annealed to a complementary template (with an increased FP value).

A universal FP-TDI protocol for SNP detection described by Chen (44) and Hsu (45) was applied to FVL (46), and is commercially available from Perkin Elmer (Waltham, MA; <http://www.perkinelmer.com>). In this assay, a preassay PCR amplicon is treated with alkaline phosphatase (AP) and *E. coli* exonuclease I to degrade unincorporated primers and nucleotides (Fig. 9A). Then, the samples are incubated with TDI-specific primers, DNA polymerase, and fluorescently labeled dideoxy-terminating nucleoside triphosphates

specific for each SNP, e.g., rhodamine-110-ddCTP (R110-ddCTP) and tetramethyl-6-Carboxyrhodamine-ddTTP (TAMRA-ddTTP). The 3'-end of the TDI primer anneals immediately upstream of the SNP, and is extended by one nucleotide that is complementary to the target SNP. Incorporation of a labeled dideoxynucleotide into the primer increases the effective molecular weight fluorophore and FP value (Fig. 9B).

The FP-TDI assay is simple (e.g., no separation of free from bound label is needed) and low cost (e.g., no lengthy manufacturing costs for new targets, no labeled primers, inexpensive equipment), and single-base extension assays are easily optimized. FP-TDI assays can be

Fig. 9. Fluorescence polarization detection template-directed dye-incorporation assay (FP-TDI). (A) PCR amplification of target is followed by degradation of primers and enzymes. Then, the amplicons are incubated with a TDI primer having a 3'-end immediately upstream from the SNP site, allele-specific dye terminators (R110-ddCTP and TAMRA-ddTTP), and DNA polymerase. The genotype is determined by monitoring changes in FP as a function of wavelength. (B) Scatter plot for Factor V genotyping. Diamonds represent FVL homozygotes detected by TAMRA-dye, light-grey squares represent FV WT homozygotes detected by R110 dye, triangles represent FVL heterozygotes detected by incorporation of both TAMRA and R110, dark-grey squares represent indeterminate samples, and stars represent negative controls. Figure taken from Freeman et al. (46).

adopted to low volume SNP laboratories with just a FP reader or scaled up to high-throughput screening with full automation. A study on 92 genomic samples using FP-TDI genotyped seven polymorphisms associated with cardiovascular diseases (β -fibrinogen C148T, Factor VII R353Q, Factor XIII Val34Leu, Glycoprotein IIIa PlA1/A2, MTHFR C677T) or thromboembolic disorders (prothrombin G20210A and FVL). The false-negative rate for detecting the FVL mutation was 2.2% when compared with sequencing. The false-negative rates

for the other mutations tested were: 3.3% for prothrombin G20210A, 2.0% for β -fibrinogen C148T, 2.2% for Factor VII R353Q, 1.1% for Factor XIII Val34Leu, 3.3% for Glycoprotein IIIa PlA1/A2, and 0% for MTHFR C677T. Another study genotyped DXS17 and D18S8 in 78 samples to validate the FP-TDI assay, and concluded that the false-negative rate was $\sim 5\%$ with all failures attributable to suboptimal pre-PCR, and the false-positive rate is $\sim 1.5\%$ with contamination being the cause (47).

PCR assays

Amplification-refractory mutation system

The amplification-refractory mutation system (ARMS) assay was first described in 1989 for detecting α -1 antitrypsin deficiency (48). An ARMS assay for FVL detection was developed with two separate PCR reactions, each using an SNP-specific primer in combination with the same locus-specific primer (49) (Fig. 10A). A mismatch at the 3'-end of the SNP-specific primer interferes with the DNA polymerase extension reaction. The amplicon, a 231 bp product, is identified by gel fractionation or automated analysis (50) (Fig. 10B). The Stagen kits (Stago, Asnieres, France; <http://www.stago.fr>) for FVL and prothrombin G20210A amplify β -2 microglobulin and an unconnected region of FV or prothrombin genes as controls (51). The ARMS assay results on 100 whole blood samples were identical with RFLP results (52). Similarly, the ARMS-method was concordant with RFLP-results on 196 DNA samples (53). In another study, the ARMS method and RFLP were compared by genotyping 67 FV WT and 108 FVL heterozygote samples; the results were identical except for one sample which was typed as heterozygote by RFLP, but FVL homozygote by ARMS (54). The ARMS assay can be designed to test for a single SNP in a multiallelic system. The main advantage of ARMS is that the amplification and diagnostic steps are combined.

Melting curve analysis using real-time quantitative PCR

Real-time quantitative PCR (Q-PCR) is the state-of-the-art technology for the nucleic acids detection and quantification, ubiquitous in research, and increasingly impacting the diagnostic field, because such assays provide greater flexibility than the gel-based PCR end-point assays. In a real-time PCR assay, a positive reaction is detected by accumulation of a fluorescent signal. The cycle threshold (Ct) or crossing point (Cp) is defined as the number of cycles or minutes required for the fluorescent signal to rise above the background signal level. Ct times are inversely proportional to the amount of target DNA in the sample. Today there is about ~20 different Q-PCR detection systems available (<http://www.gene-quantification.info>).

Melting curve analysis using FRET is based on the interaction between the electronic excited states of two dye molecules. Excited energy is transferred from one (the donor) dye molecule to a second (the acceptor) without emission of a photon, when the emission energy of the former overlaps the excitation wavelength of the latter. The transfer is distance dependent, i.e., the donor and the acceptor must be within 10–1,000 nm. FRET Q-PCR with melting curve analysis based genotyping using allele-specific

fluorescence to differentiate PCR products by T_m characteristics, and was shown to be as reliable as RFLP analysis (54,55).

In 2003, Roche Diagnostics (Indianapolis, IN; <http://www.roche-diagnostics.us/>) introduced FRET Q-PCR with melting curve analysis-based genotyping of FV and Factor II that became the first DNA-based tests to be approved by the U.S. FDA. In this assay, the appearance of a specific PCR product is monitored by annealing of fluorescent-labeled oligonucleotide hybridization probes adjacent to each other on the same strand of product (Fig. 11A). A longer probe functions as the “anchor” and “donor” probe and is labeled at the 3'-end with dye Red 705 or dye Red 640. The “acceptor” ASO probe labeled at the 5'-end with dye LC-Red 705 or dye LC-Red 640 is complementary to the SNP site, and has a 3'-phosphorylated end to prevent elongation by polymerase. The intensity of fluorescence emission from the acceptor probe is a function excitation intensity, PCR product sequence and amount, and temperature. Fluorescence decreases as the probes melt off the PCR amplicon. The data represented as the negative first derivatives ($-dF/dT$) reveal high resolution T_m s allowing SNP distinctions (Fig. 11B).

Simultaneous detection of both the FVL mutation and the prothrombin G20210A mutation is possible with Roche Diagnostics' assay, using two unlabeled amplification primer sets and two pairs of differentially labeled fluorescently oligonucleotide probes for each SNP (56). A triplex “asymmetric” assay using three different fluorescence label sets simultaneously detects FVL, the prothrombin G20210A mutation, and the FVII C46T mutation. In this assay, fluorescence emission obtained from different channels are compensated for by the use of asymmetric concentration of primers, i.e., using an excessive amount of amplification primers allows the preferential synthesis of the reverse strand complementary to the hybridization probes, which causes a significant increase of fluorescence intensity on the FRET-based reaction and a significant increase of the specificity during melting analysis. An efficiency gain is obtained in the melting curves for each target without disturbing the fluorescence emission produced by other channels and without significantly increasing the Cp obtained from each amplification reaction. The triplex assay showed full concordance with RFLP and sequencing analysis when tested on 67 samples (57). The melting curve analysis using FRET Q-PCR is suitable for clinical laboratories with medium or high throughput.

Helicase-dependent amplification

Helicase-dependent amplification (HDA) is an isothermal nucleic acid amplification technique (58). HDA uses two enzymes, a DNA helicase that denatures

Fig. 10. Amplification refractory mutation system (ARMS) PCR. (A) Two separate allele-specific reactions are done. Each primer has a 3'-terminal residue specific to one SNP plus one destabilizing 3'-end mismatch at position-3. Gel electrophoresis reveals single products for FVL homozygote or FV WT amplification, whereas heterozygote amplification will be seen in both reactions. (B) ARMS results on three whole blood samples. Samples 1, 2, and 3 are homozygous for FV WT, heterozygous, and homozygous for FVL, respectively. A = FV WT and B = FVL PCR reactions. The control band is the internal cystic fibrosis PCR product. Figure modified from Scobie et al. (52).

double-stranded DNA in a 3' to 5' direction and DNA polymerase. Biohelix Corporation (Beverly, MA; www.biohelix.com/) markets a for-research-use-only FVL genotyping assay based on this technique using differentially labeled allele-specific primer and one common biotin-labeled primer which is annealed to each single strand (Fig. 12A). The wild type primer has a 5'-terminal G labeled with fluorescein (*F*) and the FVL primer has a 5'-terminal A labeled with digoxigenin. Similar to PCR, amplification occurs through a DNA (here, Bst) polymerase reaction; however, no denaturation is needed because helicase denatures the products. After HDA, amplicons are captured on a test strip with arrayed antibodies specific for the different labels.

Biotin in each amplicon binds streptavidin-conjugated color substrate (Fig. 12B). Seven clinical samples from Lahey Clinic (Burlington, MA), four FV WT, two FVL

heterozygotes, and one FVL homozygote, have been tested with 100% correlations with known genotypes determined by RFLP. Advantages include the fact that this procedure is isothermal (i.e., no need for a thermocycler) and detection uses a disposable strip. The method could be adapted for multiplexing and large-scale testing if assembled with automated data collection.

DNA Sequencing

Dideoxy sequencing

Direct sequencing of DNA detects all SNPs and is considered the "gold standard" for molecular diagnostics (59). The Sanger technique uses 2',3'-dideoxynucleotide phosphates (ddNTPs), molecules that differ from deoxynucleotides by having a hydrogen atom attached to the 3'-carbon rather than an OH group to terminate DNA

Fig. 11. Melting curve analysis using real-time quantitative PCR (Q-PCR). (A) During the PCR annealing step, two differentially labeled fluorescent oligonucleotides hybridize side-by-side on the target DNA. The excitation of fluorescein (F) on the donor probe leads to emissions that excite the receptor Red 640 on the adjacent probe. (B) Melting curve analysis of the three possible FV genotypes and a nontemplate control. Shown is the first negative derivative of the fluorescence with respect to temperature $[-d(F)/dT]$. Melting curve analysis indicates that the fully matched duplex (FV WT) has a higher T_m than a mismatched duplex with FVL SNP. Heterozygous samples have two peaks each at the temperatures of the respective homozygous samples. Figure modified from package insert LightCycler Factor V Leiden, Primer/Hybridization Probes Reagent for the detection of the Factor V Leiden point mutation in the human Factor V gene Version 4, April 2003, Cat. No. 3 028 526 (Roche, Applied Sciences, Penzberg, Germany).

Fig. 12. Helicase-dependent amplification (HDA). (A) Helicase enzyme unwinds double-stranded DNA to single strands. One fluorescein (F)-labeled FV WT primer or digoxigenin (D)-labeled FVL primer and a common biotin (b)-labeled primer anneal to complementary single strands. Polymerase extends the 3'-ends of each primer with helicase HDA denaturing the double-stranded template, resulting in exponential amplification of the target and double-stranded products. The amplicons are captured by anti-D and anti-F-specific antibodies. (B) Biotin (b) binds SAV conjugates generating a colored C (FV WT) or T (FVL) line. Figure modified from www.biohelix.com/IsoAmp_Factor_V_Leiden_SNP_Det.asp

chain elongation. The reaction contains the target, primer, and ddNTP chain terminators specific for A, C, G, or T. The ratio of dideoxy to deoxy nucleoside triphosphate determines the distribution of dideoxy into newly synthesized DNA. The 3'-terminal ends of

the truncated fragments in a reaction will be the same ddNMP. Usually, Sanger sequencing requires three steps: amplification of the target, purification of the resulting PCR amplicons to remove unincorporated deoxynucleotide triphosphates (dNTPs) and primers,

Fig. 13. Dideoxy sequencing. (A) Dye-labeled chain terminators ddNTPs (differentially labeled with fluorescent dyes, each with different wavelengths of fluorescence and emission), dNTPs, polymerase and sequencing primers are added to a purified and denatured PCR amplicon. The dNTPs are incorporated according to template sequence and ddNTPs are incorporated randomly into the corresponding position and will terminate sequence. (B) Sequencing results show chromatogram of a FV WT homozygous sequence and a FVL heterozygous sequence. The red arrow indicates the FVL mutation site. Figure taken from Murphy et al. (60).

and a sequencing reaction to generate sequencing products (Fig. 13A, B). With this technique, multiplexing is limited as well as the ability to detect a heterozygous SNP in a PCR product.

Murphy et al. described a sequencing method AmpliSeq that reduced the three separate steps to a single reaction, by using two oligonucleotide primers at unequal molar ratio, higher than usual dNTPs, and 35–45 thermal cycles combining amplification and cycle sequencing reactions (60). Analysis of results at different cycle numbers suggest that these reactions are dominated by PCR during early cycles and sequencing occurs after 20 cycles. The AmpliSeq results correlated with the results of the RFLP assay.

Pyrosequencing

Today, pyrosequencing is pushing development of much more flexible formats that avoid gel-based fractionations, promote automation, and substantially increase throughput. Pyrosequencing, first described in 1998 by Ronaghi

et al., detects the release of pyrophosphate that accompanies the incorporation of each nucleotide into the growing DNA strand by DNA polymerase (61). In pyrosequencing, four enzymes (DNA polymerase, ATP sulfurylase, luciferase, and apyrase) are used and, usually, the template–primer complex is immobilized to a surface to facilitate cycling (Fig. 14A).

For each cycle, a DNA polymerase extension reaction is carried using a single dNTP precursor, adenosine 5'-phosphosulfate (APS), and luciferin. ATP sulfurylase (generates ATP from pyrophosphate and APS) and an ATP-dependent luciferase reaction generates oxyluciferin and light from luciferin.

After each cycle, apyrase degrades unincorporated dNTPs and ATP resetting the system. The incorporation of each nucleotide is tested sequentially, and the time-dependent appearance of the visible light (a “pyrogram”) indicates the incorporation of a specific base (Fig. 14B). At least for small number, repeating nucleotides are detected by quantitative differences in signal intensity.

Fig. 14. Pyrosequencing. (A) PCR amplicon is denatured and hybridized to magnetic beads with single-stranded DNA. Then, a primer is hybridized to the captured single strand, and the sample is incubated sequentially with each of the four deoxyribonucleotide triphosphates (dNTPs) and needed enzymes. When dNMP is incorporated into the primer, pyrophosphate (PPi) is released and converted to ATP by APS. The ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates light. (B) The plot of time vs. intensity of light produced is a PyrogramTM. Top: sequence around the mutation site of the different genotypes. Bottom: the raw data obtained by pyrosequencing for respective genotypes when nucleotide dispensation order is CTGCGCTA. The grey background shading indicates the distinguishing peaks that will differ between alleles. Figure modified from Holmberg et al. (62).

Holmberg et al. developed a pyrosequencing protocol for parallel genotyping of 11 genes, including FVL (62) (Fig. 14). Subnanogram amounts of genomic DNA from 480 individuals were amplified with PCR using a biotinylated primer. Single biotinylated

double-stranded amplicons were immobilized on a streptavidin-coated magnetic bead that was heated to remove the nonbiotinylated strand. Then, a sequencing primer was added and the sample incubated with the four enzymes and substrates.

The order of nucleotide dispensation for FVL was CTGCGCTA. If the added nucleotide is target complementary, it will be incorporated and light will be generated, but if the added nucleotide does not form base pair with the target template, no light will be produced. Each pyrogram peak represents the sum of nucleotide incorporation at each nucleotide dispensation step into all DNA templates in the same reaction mixture.

Cleavase

Invader assay

The Invader Assay was first described in 1999 (63) and was applied to FVL detection in the same year (64). The technique uses signal rather than product amplification, and depends on an enzyme, cleavase, to make endonucleolytic breaks of DNA at a tripartite structure (Fig. 15A). Each Invader assay uses two oligonucleotides: an Invader and an allele-specific probe. The Invader probe is complementary to the target sequence and has a 3'-terminal nucleotide that is SNP specific. An allele-specific probe is partially complementary to the target sequence, consisting of a 3'-complementary region and a 5'-noncomplementary end. In the primary reaction, the Invader probe binds to the 3'-end of target, forming a one-base overlapping structure at the SNP. In the primary reaction, the allele-specific probe binds at the SNP nucleotide site, forming a 5'-flap. Cleavase hydrolyzes the allele-specific probes at the unique trinucleotide SNP site, releasing 5'-flaps containing the nucleotide complementary to the SNP. The number of flap regions released is relative to the amount of target in the sample. The released flap regions from the primary reaction serve as Invader probe in a secondary tripartite-overlapping cleavage reaction on a labeled FRET probe (Fig. 15A). Cleavage of the FRET probe separates the donor (FAM) from the acceptor dye (Eclipse). In the absence of match between ASO and target in the primary reaction, no complex is formed and cleavage cannot occur (Fig. 15C). Using two different 5'-flap sequences with target complementary 3'-arm and FRET probes with nonoverlapping fluorophores allows for two distinct sequences to be detected in a single reaction. Third Wave Technologies (Madison, WI; www.twt.com), now Hologic Inc. (Bedford, MA; www.hologic.com), markets a universal Invader-based assay for SNPs, including FVL. The assay includes a DNA denaturation step, followed by addition of the probes and enzyme and isothermal incubation for 4 hr. Studies have indicated 99.9% concordance of the Invader methodology with standard techniques, such as allele-specific PCR, RFLP, and sequencing (65).

A primary benefit of using the Invader methodology is that no preassay amplification of the target DNA is required. Although elimination of preassay amplification eliminates one possible risk of contamination, analysis of low DNA amounts may need concentration by vacuum centrifugation or ethanol precipitation; hence, raising the possibility of contamination. Other negatives to the method include the requirement for a larger amount of starting DNA, and sometimes it is problematic to analyze heterozygous samples.

Ligase

Ligase-mediated genotyping was first described in 1988 (66). Today, this method has been adapted for studies on DNA sequencing (67), in situ protein detection) and analysis of macromolecular interactions (68).

Oligonucleotide ligation assay

In this assay, gene-specific PCR amplicons are denatured and hybridized to a 3'-biotinylated probe having a target SNP at the 5'-terminal position (69) (Fig. 16A). A 3'-digoxigenin-labeled reporter oligonucleotide serves as a common probe in a two-step ligation reaction. After ligation, the biotinylated oligonucleotides are captured on immobilized streptavidin and the unligated digoxigenin-labeled oligonucleotides are washed away. Then, signal is obtained by adding AP-conjugated anti-digoxigenin antibody, which converts formazan dye to the chemiluminescent substrate (Fig. 16B). Results of 20 samples assayed for FVL with this assay correlated 100% with results obtained by RFLP.

In 1997, an ELISA-based oligonucleotide ligation assay (OLA) for FV genotyping of 320 samples using lanthanide and biotin-labeled probes was described (70). The FV WT probe and FVL probe was labeled with samarium and europium, respectively. The results correlated with results obtained by RFLP for all genotypes: 44 FV WT homozygotes, 3 FVL homozygotes, and 44 FVL heterozygotes. ELISA-based OLAs can be automated because they are performed in microtiter wells and avoid the use of centrifugation or gel electrophoresis. Also, these assays are relatively inexpensive without the need for costly equipment and technical expertise. Like all ligation assays, the potential disadvantage is that the presence of other mutations around the ligation site may result in incorrect results.

Ligase-mediated rolling circle amplification

Rolling circle amplification (RCA) is an isothermal method that uses circular templates (71). Other techniques that utilize circular probes, called padlock

Fig. 15. Invader assay. (A) An allele-specific (here, FV WT is shown) Invader probe hybridizes to the target to form an overlapping tripartite structure at the SNP site. The tripartite site is recognized and cut by cleavase, releasing the 5'-flap-sequence of the Invader probe. The released flap serves as an Invader probe in a second cleavage reaction. The secondary hairpin target has a FAM-labeled 5'-end that is quenched by Eclipse, a non-base pairing acceptor dye replacing the base at position-4. The 3'-ends of the FRET probe is blocked with a hexanediol group (hex) to reduce background signal from the reaction. Cleavage separates the fluorophore from the quencher on the secondary target. (B) Distribution of ratios from the Invader assay results for FV WT, FV WT/FVL, and FVL genotypes. Using a WT probe, the upper limit classifying a sample as FVL was set to 0.25, although no FVL homozygous had a ratio more than half that value. To prevent uncertainty in discriminating between FV WT and heterozygous genotypes, a narrow range $\geq 3 < 5$ was set at classifying a sample as equivocal. The range of classifying a sample as FVL heterozygous were $\leq 0.25 - \leq 3.0$, but with the actual range of ratios for samples of 0.49–2.19. The values for FV WT homozygous was ≥ 5 , for FVL heterozygote $\geq 25 < 3$, and for FVL homozygous < 0.25 . Figure modified from Ledford et al. (87). (C) When the tripartite invasive structure in the first step is not formed, as in the case of FVL target tested with a FV WT-specific probe, cleavage will not occur in either the primary or the secondary reactions.

Fig. 16. ELISA-based oligonucleotide ligation assay (OLA). (A) A PCR amplicon denatured and hybridized with two oligonucleotides. A biotin (b)-labeled oligonucleotide is specific for the FVL allele and the digoxigenin (D)-labeled oligonucleotide is a common probe. After ligation, the doubly labeled oligonucleotide is captured by binding to immobilized SAV. Alkaline phosphatase (AP)-conjugated antibody anti-D antibody is added. With the FVL template, a mismatch between the FV WT- and FVL-specific oligonucleotide prevents ligation. (B) Segment of a 96-well microtiter plate showing substrate color development using FV WT and FVL probes with FV WT, FV WT/FVL, and FVL targets. Figure modified from Benson et al. (70).

probes or open circular probes (OCPs), use ligation reactions (72).

An OCP has end segments, complementary to a target sequence, connected by a linker sequence that is not complementary to genomic DNA. The hybridization of the end segments to target DNA sequence promotes end joining by DNA ligase to form a closed circularized

OCP, whereas mismatching ends prevent ligation. The closed circular OCPs can be used as a template in RCA (73). L-RCA is used in a variety of applications, including virus genome amplification (74), cloning (75), RNA detection (76–78), sequencing (78), ELISA (79), and immunohistochemistry (80). The most common applications of OCPs in SNP diagnostics take advantage

Fig. 17. L-RCA using hairpin-OCP. (A) (1). Ligation using a hairpin open circular probe (OCP). The structure of the hairpin probe is maintained in a self-annealed state unless the hairpin sequence encounters its complementary sequence. Only base pairing between the target sequences is sufficient to disrupt the hairpin. (A) (2). RCA begins when the Q-PNA primer (P1) anneals to the circularized OCP and polymerase copies the circular template sequence producing single-stranded binding sites for the allele-specific primer (P2). Each turn of displacement synthesis around the circle exposes another P2-binding site, and the resulting synthesis from the annealed P2 primers results in double-stranded linear products. Denaturation of the Q-PNA separate the fluorescent label on the DNA from the quencher on the PNA, producing fluorescent signal specific for the P1/circle pair. (B) Mismatch between FVL target DNA. OCP promotes the formation of an intramolecular 3'-hairpin. During RCA, the hairpin is extended by polymerase, resulting in an extended hairpin with minimal single strandedness, that is unlikely to participate in faulty priming reactions and misamplification. (C) Real-time and end-point analysis. Real-time assay: the fluorescence vs. time plotted for reactions with 48 samples. The genotype for each sample was determined by measuring Ct for each sample. End-point assay: RCA reactions were incubated at 60°C for 30–40 min. The genotype of each sample was determined automatically, using a modified fuzzy c-means clustering algorithm which groups the data into three genotypes plus a negative control, and assigns a confidence level (0–100%) to each genotyping call. Figures taken from Alsmadi et al. (81).

C

Real Time Assay

End Point Assay

Fig. 17. Continued.

of the mismatch-discriminating properties of DNA ligase and/or polymerase.

In L-RCA, the OCP-termini anneal on target site, and the base complementary to the SNP is usually at a 3'-end. Ligation occurs when the 3'-terminal nucleotide is complementary to the target SNP, then the circularized template is amplified by RCA.

Alsmadi et al. designed a FVL L-RCA assay using an OCP that forms a 3'-end hairpin in the absence of a complementary sequence (81) (Fig. 17A). One primer has a fluorescent moiety (either FAM or Cy3) and a 5'-end of 13 nucleotides complementary to the 5'-end of linker region plus 3 nucleotides in the complementary 5'-arm of the OCP. A quencher-labeled peptide nucleic acid (Q-PNA) probe with a C-terminal quencher (dabcyl) composed of bases complementary to the 13 nucleotide tag hybridizes to the tag sequence and

quenches the fluorescence from the primer. PNA is a DNA analogue with a neutral peptide rather than phosphodiester backbone that anneals to a complementary DNA with higher affinity than the complementary DNA (82). The second primer has the same sequence as the 3'-target-specific region of the OCP and is complementary to the extended product of the first primer. Amplification is monitored in real-time, because the signal from unused fluorescently labeled oligonucleotide primers is quenched by hybridization to the Q-PNA and fluorescent signal is produced when the DNA polymerase displaces dabcyl-containing Q-PNA from the fluorescent label on the RCA product. The DNA polymerase strand displacement activity exposes the primer-binding sites for further exponential cascade amplification. The end product of the reaction is a double-stranded amplicon with one strand fluorescently

Fig. 18. (A) L-RCA using GAP-filling oligonucleotides. The system comprises an open circular probe (OCP) with two target complementary regions at the 5'- (dotted black) and 3'-ends (striped grey) that hybridize to the target nearby but not adjacent to each other. The gap is filled by ligation to both OCP ends of two short intervening GAP-filling oligonucleotides (dotted grey and solid grey), whereas the internal linker region forms a hanging circular structure. The closed OCP is amplified with RCA. One primer annealed to the single-stranded linker region of the OCP creates a linear concatemer of the closed circular OCP. A second primer has a 3'-arm at the SNP site and a hairpin loop structure at the 5'-end bringing Cy3 fluorophore and quencher, Dabcyl (Q), to close proximity. The second primer anneals once to each tandem repeated OCP. (B) Results of FVL detection with L-RCA using GAP-filling oligonucleotides: four replicates of FV WT/FV WT, FV WT/FVL, FVL/FVL genomic DNA, and nontemplate controls were tested. Amplification was determined by automated calculation by the second derivative maximum method where the Ct is defined as the maximum of the second derivative from the fluorescence curve of the sample.

labeled. In the absence of ligation, the 3'-end hairpin is extended by DNA polymerase to form a double-stranded linear structure resistant to RCA (Fig. 17B). This technique was used for FV genotyping in 105 FV WT, 98 FVL heterozygote, and 13 FVL homozygote samples (Fig. 17C). The accuracy for each single genotyping assay was 99.5% and the observed genotyping accuracy triplicate testing was 100% when compared with RFLP analysis.

We developed an L-RCA system for FVL detection (unpublished). The OCP had a 17- and 15-base sequence at the 3'- and 5'-end, respectively, complementary to the target (Fig. 18A). In addition, two eight-base gap-filling oligonucleotides hybridized to the target between the OCP end sequences. The SNP is at the 3'-end of the oligonucleotide nearest the 3'-end of the OCP. Ligations at three points are required to form a closed circular OCP: one between the gap-filling oligonucleotides and one each between each of the gap-filling oligonucleotide and the OCP ends. The DNA circle serves as template for an exponential or hyperbranching RCA reaction, using one primer complementary to the single-stranded linker region between the complementary end sequences and a second primer is an "Amplifluor™"-primer, with a 5'-hairpin loop structure having a fluorophore (Cy3) and quencher (Dabcyl) (Fig. 18A). When the OCP is circularized, RCA creates linear concatemers of the OCP, to which the second primer binds. Fluorescence is detected when the incorporated Amplifluor primer stem is denatured by DNA polymerase activity. This investigational assay was tested on apparently healthy DNA, FV-deficient DNA (FVL), and FVL heterozygous DNA (Coriell Cell Repositories, Camden, NJ). The cutoff value was set so that if a sample had a Ct of 20 cycles or above it was considered negative for FVL. All replicates of the FVL and FV/FVL had a Ct around 15 cycles, whereas the FV WT and nontemplate controls had a Ct of > 50 (Fig. 18B).

L-RCA is an investigational method suited for automated, high-throughput applications. The amplification step is performed at one temperature. However, today, preceding denaturation and ligation are done at different temperatures; therefore, the assay as a whole is not isothermal. Attempts to develop a complete isothermal L-RCA technique where ligation and RCA are integrated into one step are in progress.

DISCUSSION

The only clinically used protein-based test is the functional coagulation test, which measures the time it takes for patient blood to form a clot. The test using APC have been consistently used to detect coagulation

deficiencies long before DNA-based techniques were available. Functional techniques can greatly reduce the time needed for diagnosis; however, the results should only be considered as presumptive because both false positives and negatives are possible and presence of the SNP is not certain. The recently described peptide antibody immuno-sensor technique for FVL is investigational (24), but might lead to protein-based ELISA test relying on the FV proteins immunological properties where the developed antibodies truly discriminate between proteins.

In order to improve accuracy and quality of genetic testing of FV, the first International Genetic Reference Panel for FVL was established for a panel of three genomic DNA samples (FV wild type, FVL homozygote, and FVL heterozygote) in 2004 (83). The recommendation was approved by the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis and the Expert Committee on Biological Standardization of the World Health Organization. Eight hundred and fifty-nine genotyping tests were carried out on the panel with an error rate of 0.7%.

Many of the clinically used assays to detect FVL have low throughput and are laborious. Efforts to create high-throughput automated assays allowing testing of a large number of samples have made significant advances in the past decade, but many today lack validation in a clinical setting. Although not one-step, many laboratories perform FVL testing within a single day (sample collection to extraction to PCR to analysis to report). High-throughput methods are only needed if sample volume is sufficient; otherwise, a high-throughput method is wasteful. Often with genetic testing, testing a high number of samples daily/weekly/monthly is not realistic. Medicine is moving toward same-day, one-step, on-site protocols for identification of all genetic disorders as well as pathogens. Currently, no test for detecting the FVL mutation exists that fulfill those criteria.

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