

Technical standards and guidelines: Venous thromboembolism (Factor V Leiden and prothrombin 20210G>A testing): A disease-specific supplement to the standards and guidelines for clinical genetics laboratories

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The ACMG Laboratory Quality Assurance Committee has the mission of maintaining high technical standards for the performance and interpretation of genetic tests. In part, this is accomplished by the publication of Standards and Guidelines for Clinical Genetics Laboratories, the latest edition of which is maintained online at <http://www.acmg.net>. The committee also reviews the outcome of national proficiency testing in the genetics area and may choose to focus on specific diseases or methodologies in response to those results.

Because the test for hypercoagulability is one of the most frequently ordered molecular genetic tests and one for which clinical guidelines have recently been issued by the ACMG,¹ the Molecular Subcommittee of the Laboratory Quality Assurance Committee selected two mutations associated with increased risk for occurrence/recurrence of venous thrombo-

sis—the factor V Leiden and the prothrombin 20210G>A (factor II) mutations—as important topics in a series of supplemental sections.

This document follows the outline format of the general Standards and Guidelines for Clinical Genetics Laboratories. It is designed to be a checklist for genetic testing professionals who are already familiar with the disease and the methods of analysis.

FV/PT 1 INTRODUCTION

Disease-specific statements are intended to augment the current general ACMG Standards and Guidelines for Clinical Genetics Laboratories. Individual laboratories are responsible for meeting the CLIA/CAP quality assurance standards with respect to appropriate sample documentation, assay validation, general proficiency, and quality control measures.

FV/PT 2 BACKGROUND ON FACTOR V LEIDEN AND PROTHROMBIN 20210G>A MUTATIONS

FV/PT 2.1 Gene symbol/chromosome locus. Factor V Leiden: F5; 1q23. Factor II: F2; 11p11-q12.

FV/PT 2.2 OMIM number. Factor V Leiden: 227400. Factor II (also referred to as Prothrombin): 176930 (See Table 1)

FV/PT 2.3 Brief clinical description. The annual incidence of venous thromboembolism (deep vein thrombosis and pul-

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Table 1

	Factor V Leiden	Factor II, Prothrombin
Gene symbol	F5	F2
Chromosome locus	1q23	11p11–q12
OMIM number	227400	176930

monary embolism) is approximately 117 per 100,000 persons or about 1 per 1,000 person-years.^{2,3} However, the risk for venous thromboembolism is related to age, with the majority of the disease occurring in the older age groups. The risk of thrombosis before age 40 is approximately 1 in 10,000 persons per year, rising to 1 in 100 persons per year after age 75.⁴

Factor V Leiden is a missense mutation in the clotting factor V gene.⁵ The mutation is commonly referred to as R506Q. This results in a protein product that is resistant to cleavage by activated protein C (APC), a key step in the anticoagulation system.⁶ The resulting phenotype of APC resistance is characterized primarily by increased risk of venous thrombosis, but has also been associated with recurrent pregnancy loss and complications, as well as myocardial infarction in young adult female smokers. For more information, see the ACMG Consensus Statement on Factor V Leiden Mutation Testing.¹

The prothrombin 20210G>A mutation in the factor II gene was first described in patients with a family history of thrombosis.⁷ The prothrombin 20210G>A mutation is present in approximately 0.7% to 4.0% of the Caucasian population⁸ and in approximately 6% of patients with a first venous thromboembolism.⁷

Martinelli et al.⁹ found that the 20210G>A mutation in the factor II gene and the factor V Leiden mutation are associated with “idiopathic” cerebral vein thrombosis. The use of oral contraceptives was also strongly and independently associated with the disorder. The presence of both the prothrombin 20210G>A mutation and oral contraceptive use raised the risk of cerebral vein thrombosis further. The factor V Leiden and prothrombin 20210G>A mutations previously had been known to be common genetic determinants of deep vein thrombosis of the lower extremities. The findings of Martinelli et al.⁹ represent a prime example of the interaction of endogenous (genetic) and exogenous factors in causation of disease.⁵

De Stefano et al.¹⁰ examined the relative risk of recurrent deep venous thrombosis in their studies. The authors found that while patients who were heterozygous for factor V Leiden had a risk of recurrent deep venous thrombosis similar to that among patients with no known mutations in either factor II or factor V (relative risk, 1.1), patients who were heterozygous for both factor V Leiden and prothrombin 20210G>A had a 2.6-fold higher risk of recurrent thrombosis than did carriers of factor V Leiden alone. The resulting phenotype is characterized primarily by increased risk of venous thrombosis, but has also been associated with recurrent pregnancy loss and complications, as well as myocardial infarction in young adult female smokers.

Venous thromboembolism is a complex disease involving several genetic abnormalities besides factor V and factor II.

Antithrombin deficiency, protein C deficiency, APC resistance, and mutations in methylene tetrahydrofolate reductase (MTHFR), alone or in combination with factor V Leiden and/or prothrombin 20210G>A mutations, are associated with venous thromboembolism.¹¹ Independent environmental factors contributing to venous thromboembolism include, but are not limited to the following: smoking, male sex, confinement to a hospital or nursing home, older age, trauma sufficient to require hospitalization, malignant neoplasm, neurologic disease with chronic extremity paresis, superficial vein thrombosis, and prior central venous catheter or transvenous pacemaker.² Additional risks for women include pregnancy and use of oral contraceptives, estrogen replacement therapy, tamoxifen, and raloxifene.^{12,13} This document covers only testing for the factor V Leiden and prothrombin 20210G>A mutations.

FV/PT 2.4 Mode of inheritance. Both the factor V Leiden mutation and the prothrombin 20210G>A mutation exhibit semidominant expression in that both heterozygotes and homozygotes are at increased risk of occurrence/recurrence of venous thrombosis. The relative risk for venous thrombosis associated with the factor V Leiden mutation in the absence of other acquired or environmental predispositions is approximately 4- to 7-fold for heterozygotes and 80-fold for homozygotes.¹⁴ The relative risk for venous thrombosis associated with the prothrombin 20210G>A mutation in the absence of other acquired or environmental predispositions is approximately 2- to 4-fold for heterozygotes.¹⁵

FV/PT 2.5 Gene description/normal gene product

FV/PT 2.5.1 Factor V. The gene product is factor V, an important component of the coagulation cascade, which, in association with factor Xa, activates prothrombin to thrombin.

FV/PT 2.5.2 Factor II. The gene product is factor II, an important component of the coagulation cascade. It is a vitamin K-dependent protein that participates in coagulation and its regulation. Prothrombin participates in the final stages of the blood coagulation cascade, where it is converted to thrombin in the presence of factor Xa, factor Va, calcium ions, and phospholipid.

FV/PT 2.6 Mutational mechanism/abnormal gene product

FV/PT 2.6.1 Factor V Leiden. The Leiden allele of the factor V gene contains a G→A substitution at nucleotide 1691, producing a missense mutation that substitutes glutamine for arginine at amino acid residue 506 (R506Q) in the protein product. The R506Q site is one of the APC cleavage sites in the factor Va molecule.^{16,17}

FV/PT 2.6.2 Factor II (Prothrombin 20210G>A). The prothrombin 20210G>A mutation is located in the 3' untranslated region of the factor II gene. Gehring et al.¹⁸ demonstrated that the 20210G>A mutation does not affect the amount of premRNA, the site of 3' end cleavage or the length of the poly(A) tail of the mature mRNA. Gehring et al.¹⁸ determined

that the physiologic factor II 3' end cleavage signal is inefficient and that the 20210G>A mutation represents a gain-of-function mutation, causing increased cleavage site recognition, increased 3' end processing, and increased mRNA accumulation and protein synthesis.

FV/PT 2.7 Mutation spectrum

FV/PT 2.7.1 Factor V. Factor V Leiden accounts for at least 80%¹⁶ or 90% to 95% of cases of APC resistance.¹⁴ Two other mutations in the gene have been described, both at the same locus—factor V Cambridge (R306T)¹⁹ and factor V Hong Kong (R306C)¹⁷—but definite associations with venous thrombosis or APC resistance have not been confirmed for these mutations. Another variant, called the R2 allele (H1299R), appears to confer a modest additional increased thrombotic risk when present in the compound heterozygous state with factor V Leiden.²⁰ It has no clinically significant effect by itself, but in the homozygous state, it appears to increase APC resistance. It is not present in the same haplotype as factor V Leiden and so is not found in factor V Leiden homozygotes.²⁰ For routine purposes, factor V Leiden itself is the only mutation of this gene for which testing is indicated, and it is the only one covered by this document.

FV/PT 2.7.2 Factor II. The 20210G>A mutation accounts for a large proportion of reported mutations in the prothrombin gene. Although other allelic variants have been described, no others are present in frequencies high enough to warrant genetic testing. For routine purposes, prothrombin 20210G>A itself is the only mutation for which testing is indicated, and it is the only one covered by this document.

FV/PT 2.8 Ethnic association of common mutations

FV/PT 2.8.1 Factor V Leiden. The factor V Leiden mutation is most prevalent in the US and European Caucasian populations, in which the frequency of heterozygotes of approximately 5% to 7% have been reported. The frequency of homozygosity for the factor V Leiden mutation was approximately 0.02% in one study.⁸ The factor V Leiden mutation is found in 5.27% of Caucasian Americans and is progressively less common in Hispanic Americans (2.21% heterozygotes), Native Americans (1.25% heterozygotes), African Americans (1.23% heterozygotes), and Asian Americans (0.45% heterozygotes).²¹ Gregg et al.²² found very similar ethnic differences.

FV/PT 2.8.2 Prothrombin 20210G>A. The heterozygous G→A substitution at the residue 20210 polyadenylation site is reported to be present in about 2% in the general population, with an increased frequency (3.0%) in southern Europeans and a decreased frequency (1.7%) in northern Europeans. It is very rare among those of Asian and African descent.²³

FV/PT 2.9 Clinical and analytic validity

FV/PT 2.9.1 Clinical validity. The clinical validity of a genetic test defines its ability to accurately and reliably identify

individuals who have (or will develop) the disorder or phenotype of interest.

FV/PT 2.9.1.1 Clinical sensitivity. Clinical sensitivity can be defined as the proportion of individuals who have had (or will have) deep vein thrombi and who have at least one factor V Leiden or one prothrombin 20210G>A mutation (http://www.acmg.net/Pages/ACMG_Activities/stds-2002/cf.htm, Section CF 2.11.2). Clinical sensitivity is equivalent to the detection rate. Overall, the clinical sensitivity of the factor V Leiden mutation is between 20% and 50% (see Endler and Mannhalter²⁴ for review).

Among men older than 60 years of age with the first spontaneous thrombotic episode, the Physicians' Health Study found that approximately one-quarter carry one factor V Leiden allele.²⁵ Similar sensitivity (29.5%) was found in another study with 380 individuals with at least one thromboembolic event.²⁶ In addition, this allele has been found in 20% to 46% of pregnant women with venous thrombosis.^{27,28}

The clinical sensitivity of the prothrombin 20210G>A mutation varies between 5% and 19% (see Endler and Mannhalter²⁴ for review).

Data from several studies strongly suggest that the pathogenesis of venous thromboembolism is multifactorial and requires interactions between both inherited and acquired risk factors.^{24,29} Heterozygosity for the factor V Leiden or prothrombin 20210G>A mutations alone may be a relatively weak risk factor unless a second genetic risk factor or an acquired factor, such as older age, also exists.

FV/PT 2.9.1.2 Clinical specificity. Clinical specificity can be defined as the proportion of individuals who do not have or will not develop deep vein thrombosis and do not have any known mutations in the factor V Leiden or prothrombin genes (http://www.acmg.net/Pages/ACMG_Activities/stds-2002/cf.htm, Section CF 2.11.2). The false-positive rate is 1 minus the clinical specificity. Low penetrance of the factor V Leiden mutation or the prothrombin 20210G>A mutation is the main reason why clinical specificity is less than 100%. Analytic error is possible, but likely to be a much smaller factor in clinical false-positive test results.

Clinical specificity for the factor V Leiden test has not been firmly established, but can be no lower than 95% (this assumes that all 5% of the population with a mutation are clinical false positives). Similarly, the clinical specificity for the prothrombin test is likely to be no lower than 98% (if all 2% of mutation carriers are clinical false positives). Given the low penetrance of these mutations (i.e., most individuals with a mutation will not develop a venous thrombosis), these two estimates of clinical specificity are reasonably reliable.

FV/PT 2.9.2 Analytic validity. The analytic validity of a genetic test defines its ability to accurately and reliably measure a specific analyte or to identify a mutation of interest in the sample type(s) to be used clinically. Each laboratory is responsible for in-house validation of a test methodology.

FV/PT 2.9.2.1 Analytic sensitivity. The analytic sensitivity is the proportion of biological samples with a known mutation that is correctly classified as having a positive test result (see Section C8.4.1, Test Validation, in the ACMG Standards and Guidelines for Clinical Genetics Laboratories, http://www.acmg.net/Pages/ACMG_Activities/stds-2002/c.htm). The various molecular methods in use for detecting the factor V Leiden and the prothrombin 20210G>A mutations are very robust and should have sensitivities of 98% or higher.

FV/PT 2.9.2.2 Analytic specificity. The analytic specificity is the proportion of biological samples without a factor V Leiden or prothrombin 20210G>A mutation that is correctly classified as having a negative test result. (For further information, see Section C8.4.2, Test Validation, in the ACMG Standards and Guidelines for Clinical Genetics Laboratories, http://www.acmg.net/Pages/ACMG_Activities/stds-2002/c.htm.)

FV/PT 2.10 Test validation requirements. The laboratory should make every effort to satisfy the test validation criteria described by ACMG and any applicable state and federal guidelines. Information is available from ACMG and other agencies, including the New York State Department of Health (<http://www.wadsworth.org/labcert/clep/clep.html>), the National Committee for Clinical Laboratory Standards (NCCLS, MM1-A Vol. 20, #7, <http://www.nccls.org/>), and the College of American Pathologists Checklists (<http://www.cap.org/html/ftpdirectory/checklistftp.html>).

FV/PT 2.11 Special testing considerations

FV/PT 2.11.1 Diagnostic versus predictive testing. As discussed in the ACMG Consensus Statement on Factor V Leiden Mutation Testing,¹ factor V Leiden testing is predominantly used and recommended for diagnostic purposes in individuals with clinical symptoms of venous thrombosis or with recurrent pregnancy loss. Although predictive testing in asymptomatic individuals and in relatives of known factor V Leiden or prothrombin 20210G>A carriers is technically possible, its clinical utility for that purpose is markedly hampered by the low penetrance of the mutations and the appreciable risks inherent in prophylactic anticoagulant therapy.

FV/PT 2.11.2 Prenatal testing and newborn screening. As discussed in the ACMG Consensus Statement on Factor V Leiden Mutation Testing,¹ because the pregnancy complications associated (i.e., *in utero* thrombosis) with possessing the factor V Leiden mutation are generally maternal rather than fetal in origin, prenatal testing is not indicated. Similarly, because the thrombotic symptoms are of later (usually adult) onset and of low penetrance, there is no indication for newborn screening.

FV/PT 3 GUIDELINES

FV/PT 3.1 Patient guidelines: Who should be tested? The factor V R506Q (Leiden) mutation is present in approximately 5% to 7% of Caucasians and in a smaller percentage of individuals of other ethnic backgrounds. The prothrombin

20210G>A mutation is present in approximately 1% to 2% of Caucasians and in a smaller percentage of individuals of other ethnic backgrounds.²¹ Therefore, many members of the United States population would be identified as heterozygous carriers if population screening were instituted for either or both of these mutations. The risk of venous thrombosis in the general population is 1 in 1000 or 0.1%, per year.³ In heterozygous carriers of the factor V Leiden mutation, the lifetime relative risk for venous thrombosis is 4- to 7-fold.⁴ For carriers of the prothrombin 20210G>A mutation, the lifetime relative risk for venous thrombosis is 2- to 4-fold.⁷ Thus, the testing of target populations rather than the general population would appear to be the most reasonable approach.^{1,11}

Testing may have some utility in the following circumstances (these are the same as the general recommendations for testing any thrombophilia)¹:

- Age < 50, any venous thrombosis;
- Venous thrombosis in unusual sites (such as portal hepatic, mesenteric, and cerebral veins);
- Recurrent venous thrombosis;
- Venous thrombosis and a strong family history of thrombotic disease;
- Venous thrombosis in pregnant women or women taking oral contraceptives;
- Myocardial infarction in female smokers under age 50.

Other situations in which testing may be appropriate include the following:

- Venous thrombosis, age > 50, except when active malignancy is present;
- Asymptomatic relatives of individuals known to have factor V Leiden. Knowledge that they have factor V Leiden may influence management of pregnancy and may be a factor in decision-making regarding oral contraceptive use;
- Women with recurrent pregnancy loss or unexplained severe preeclampsia, placental abruption, intrauterine fetal growth retardation or stillbirth. Knowledge of factor V Leiden carrier status may influence management of future pregnancies. Known carriers of these mutations can be treated with anticoagulants during pregnancy to support a normal outcome.

Routine testing is not recommended for patients with a personal or family history of arterial thrombotic disorders (e.g., acute coronary syndromes or stroke) except for the special situation of myocardial infarction in young female smokers. Testing may be worthwhile for young patients (< 50 years of age) who develop acute arterial thrombosis in the absence of other risk factors for atherosclerotic arterial occlusive disease.

FV/PT 3.2 Definition of normal and mutation categories

FV/PT 3.2.1 Normal alleles. Normal alleles are those that do not possess the factor V Leiden R506Q mutation or the prothrombin 20210G>A mutation.

FV/PT 3.2.2 Mutant alleles. Mutant alleles are those that possess the factor V Leiden R506Q mutation or the prothrombin 20210G>A mutation.

FV/PT 3.3 Pretest considerations

FV/PT 3.3.1 Informed consent. Although formal informed consent should not be required for factor V Leiden or prothrombin 20210G>A testing, individuals being tested should be made aware that this is a genetic test and that test results have implications about risk in other family members.¹ However, laboratories need to be aware that some states may have specific requirements for informed consent.

FV/PT 3.3.2 Implications of test results. It is important for individuals testing positive for factor V Leiden and/or prothrombin 20210G>A to understand the risk implications and genetic implications of their result. The patient's physician or a genetic counselor may communicate these risks.

FV/PT 3.3.3 Pretest clinical data collection. Laboratories are encouraged to have a mechanism to collect pretest clinical information that includes the patient's date of birth, indication for testing, and specific family history of deep vein thrombosis. Routinely, laboratories also collect data regarding racial/ethnic background.

FV/PT 3.4 Methodological considerations. All general guidelines for polymerase chain reaction (PCR), gel electrophoresis, other techniques, and laboratory quality control discussed in the ACMG Standards and Guidelines for Clinical Genetics Laboratories (http://www.acmg.net/Pages/ACMG_Activities/stds-2002/g.htm, Section G7), apply to all of these assay methodologies. There are many valid methodologies for factor V Leiden and prothrombin 20210G>A analysis.

FV/PT 3.4.1 Positive controls. Some mutation-positive controls can be obtained from the NIGMS Human Genetic Cell Repository (<http://locus.umdj.edu/nigms>) as either cell lines or DNA. Genomic DNA from patients identified as being heterozygous or homozygous for the factor V Leiden or the prothrombin 20210G>A mutation can also be used as controls if consent is obtained from these patients or if the samples are anonymized. Many commercial companies provide both positive and negative controls.

FV/PT 3.4.2 Sample preparation. Most assays are amenable to the use of genomic DNA prepared from blood using a variety of extraction protocols, ranging from crude lysates to highly purified DNA.

FV/PT 3.5 PCR followed by restriction enzyme digestion

FV/PT 3.5.1 Detection of the factor V Leiden mutation. A published or in-house primer set is used to generate an amplicon that is subsequently digested with *NcoI* or another restriction enzyme that is sensitive to the mutation changing G→A at position 1691 (R506Q)³⁰ for the factor V Leiden mutation. Similar assays are available to detect the 20210G>A prothrom-

bin mutation.³¹ The DNA fragments generated by the restriction enzyme digestion are detected by gel or capillary electrophoresis. It is recommended that the DNA segments generated by PCR contain a "control" restriction enzyme site. This site serves as a control to ensure that the restriction enzyme is working correctly (http://www.acmg.net/Pages/ACMG_Activities/stds-2002/g.htm). It is also suggested that the assay be designed so that the mutation introduces a restriction enzyme site rather than eliminating a site. Control samples with a known genotype corresponding to each class (homozygous wild-type, heterozygous, homozygous mutant), as well as no-DNA controls, need to be included for each assay.

FV/PT 3.5.2 Detection of the factor II 20210G>A mutation.

A published or in-house primer set is used to generate an amplicon that is subsequently digested with *MnII* or another restriction enzyme that is sensitive to the mutation changing G→A at position 20210. The DNA fragments generated by the restriction enzyme digestion are detected by gel or capillary electrophoresis. The requirements for use of controls are the same as those described in Section 3.5.1 above.

FV/PT 3.6 Forward allele-specific oligonucleotide (ASO)

FV/PT 3.6.1 Overview. The ASO method is based upon hybridization of a labeled oligonucleotide probe containing either wild-type sequence or known mutant sequence to the target, patient DNA. A published or in-house primer set is used to generate an amplicon that includes the sequence surrounding the G→A nucleotide change at position 1691 of the factor V gene and the G→A change at position 20210 in the factor II gene. The amplicon product is bound to a membrane in an ordered array and hybridized with detection ASO probes specific for either the normal or the mutant sequence. Detection of the identity of the hybridized probe may use any of the many available labeling/detection techniques or elution of hybridized probe and identification of sequence-specific characteristics. Detection of both normal and mutant sequences in controls and patient samples is required. A number of issues must be considered in the development of this test platform.

Detailed descriptions of the factors involved, the design and labeling of ASO probes, hybridization conditions, and interpretation of results are available in the ACMG Standards and Guidelines for Clinical Genetics Laboratories (http://www.acmg.net/Pages/ACMG_Activities/stds-2002/g.htm, Section G7). No ASRs are currently available commercially.

FV/PT 3.7 Amplification refractory mutation system (ARMS)

FV/PT 3.7.1 Overview. ARMS, or amplification refractory mutation system, is the PCR equivalent of allele-specific hybridization with ASO probes. PCR reactions depend on two oligonucleotide primers that bind to the complementary strands at either end of the DNA segment to be amplified. ARMS is based on the observation that oligonucleotide primers that are complementary to a given DNA sequence except for a mismatch (typically at the 3' OH residue) will not, under appropriate conditions, function as primers in a PCR reaction.

For genotyping, paired PCR reactions are performed for each mutation tested. One primer (common primer) is used in both reactions, whereas the other is specific for either the mutant or the wild-type sequence. In principle, ARMS tests can be developed for any single base pair change or small deletions/insertions. Achieving acceptable specificity is dependent on primer selection and concentration. Use of longer primers (e.g., 30 bp vs. 20 bp) and inclusion of control reactions have been reported to improve specificity.

Home-brew primer sets must be validated to ensure desired performance characteristics and new reagent lots should be compared to a previous lot to ensure consistency in performance and robustness.

Detailed descriptions of the factors involved, the design of ARMS primers, detection conditions, control samples, and interpretation of results are available in the ACMG Standards and Guidelines for Clinical Genetics Laboratories for cystic fibrosis testing (http://www.acmg.net/Pages/ACMG_Activities/stds-2002/cf.htm, Section 3.2.3.3.1). ARMS-based ASRs are available commercially.

FV/PT 3.8 Fluorescence resonance energy transfer (FRET) assay

FV/PT 3.8.1 Overview. The fluorescence resonance energy transfer (FRET) assay represents a non-PCR-based method for the detection of known mutations.^{32,33} It utilizes allele-specific probes and a proprietary enzyme that recognizes a specific DNA structure. The initial cleavage occurs at the specific structure formed by hybridization of the target genomic DNA to a specially designed oligonucleotide probe; single-base mismatches can interfere sufficiently to diminish cleavage. In a second step, the DNA fragment released by the initial cleavage becomes the substrate for a second reaction with a different oligonucleotide probe labeled with a fluorescent reporter and quencher molecule (FRET probe). The second cleavage step releases the fluorescent probe from the adjacent quencher molecule, and multiple rounds of cleavage result in significant signal amplification. After a sufficient incubation period, samples are read in a fluorescent plate reader that can accommodate a 96-well format.

For genotyping samples, mutant and wild-type oligonucleotide probes are incubated with patient DNA samples. The actual readings are proportional to the amount of DNA and net readings are obtained by automatically subtracting the background fluorescence counts from the no-DNA control. Sample genotypes are determined by the ratio of net wild-type to mutant fluorescence counts. These net ratios are software-generated and ratios that fall within specified ranges correspond to a homozygous wild-type, heterozygous or homozygous mutant genotype. ASR and IVD reagents for this type of assay are commercially available.

FV/PT 3.8.2 Controls

FV/PT 3.8.2.1 For each mutation tested, control samples with a known genotype corresponding to each class (homozy-

gous wild-type, heterozygous and homozygous mutant), as well as no-DNA controls, need to be included for each assay.

FV/PT 3.8.2.2 Failure of any control to give a result with the expected genotype invalidates the batch and requires that the assay be repeated.

FV/PT 3.8.3 Interpretation of results

FV/PT 3.8.3.1 Sample genotype. Sample genotype is determined by the ratio of net counts of wild-type probe to net counts of mutant probe. Note that wild-type and mutant counts are obtained from separate reactions. Net counts and net wild-type/mutant ratios are automatically calculated for each sample by the accompanying software.

FV/PT 3.8.3.2 Equivocal results. Samples with results that border the specified ratio limits should be repeated to obtain unequivocal results.

FV/PT 3.9 End-point and real-time PCR analysis. These specially designed primer systems (such as TaqMan®-based and beacon-based systems) are used in end-point or real-time analysis systems to amplify and detect the mutant and normal alleles using sequence-specific hybridization based assays. Each laboratory is responsible for establishing the characteristics of the specially designed primers in the detection system used in that laboratory. Results for controls and detection cutoff limits (95% confidence) must be closely monitored to identify inadequate specimens or reaction conditions.

FV/PT 3.9.1 Melting curve analysis using FRET hybridization probes

FV/PT 3.9.1.1 Overview. There are several real-time PCR instruments. By coupling PCR with fluorescent hybridization probe analysis, these instruments can be used to detect mutations, particularly single-base mutations.³⁴ In the most common format, the PCR reaction includes locus-specific primers in addition to a pair of fluorescently-labeled oligonucleotide probes (FRET probes). One of the probes is labeled at the 3' end with fluorescein (donor dye), and the second probe is labeled at the 5' end with LC Red 640 or LC 705 (acceptor dye). The 3' end of each probe is blocked with either a dye or a phosphate group to prevent extension during PCR. The position of the probes is selected so they hybridize to the target sequence adjacent to one another, with one of the probes positioned on the mutation site. When the probes are in close proximity, the energy emitted by the excitation of fluorescein is transferred to the acceptor dye, which then emits fluorescence at a longer wavelength.

The stability of each probe/target complex as indicated by the melting temperature (T_m), depends on the length, G:C content, and sequence order. When a base mismatch is present, the thermal stability is altered. The change in stability depends on the bases involved in the mismatch, the mismatch position, and the sequence context. A melting curve of the hybridization probe fluorescence can be used to detect changes in thermal stability and therefore discriminate single base mu-

tations. During melting curve analysis, the temperature is slowly increased while the fluorescence is monitored. As the probes begin to melt from the target, the fluorescence decreases, because the probes are no longer in close proximity. If a mutation is also present, the mismatch with the probe causes the hybrid to melt at a lower temperature. The software plots the negative derivative of the fluorescence with respect to temperature. The generated peaks occur at T_m s specific for the wild-type and mutant alleles. If an additional sequence variation is present in the target, the melting profile is altered. For example, two rare polymorphisms (G1689A and A1692C) might also result in peak shifts in a factor V Leiden (G1691A) assay, but these polymorphisms can be distinguished from factor V Leiden by the T_m .

For genotyping samples, only one reaction and one set of probes are necessary. Design of PCR primers and hybridization probes follows standard methods. The assay has a large dynamic range, enabling DNA of a wide range of concentrations to be used. ASR primers and probes and factor V Leiden and prothrombin 20210G>A genotyping kits are available from the manufacturer. A number of assays using this technology for factor V Leiden and prothrombin 20210G>A genotyping have been published. The assay format can be adapted easily to mutation analysis in a number of systems.

Some systems (Taqman®) use only single-labeled probes.^{35,36} This system uses a single internal oligonucleotide probe bearing a 5' reporter fluorophore (e.g., 6-carboxy-fluorescein) and a 3' quencher fluorophore (e.g., 6-carboxy-tetra-methyl-rhodamine). During the extension phase, the TaqMan® probe is hydrolyzed by the nuclease activity of the Taq polymerase, resulting in separation of the reporter and quencher fluorochromes and consequently in an increase in fluorescence. In this technology, the number of PCR cycles necessary to detect a signal above the threshold is called the cycle threshold (Ct) and is directly proportional to the amount of target present at the beginning of the assay. The change in the amount of signal corresponds to the increase in fluorescence intensity when the plateau phase is reached. Using standards or calibrators with a known number of molecules, one can establish a standard curve and determine the precise amount of target present in the test sample.

FV/PT 3.9.1.2 Controls

FV/PT 3.9.1.2.1 Controls should be included to ensure the capability of differentiating homozygous normal, heterozygous carrier, and homozygous mutant patterns. At a minimum, this requires a heterozygous control and a negative control. When available, genomic controls are preferred over synthetic controls.

FV/PT 3.9.1.2.2 Failure of any control to give a result with the correct genotype invalidates the assay and requires that the assay be repeated.

FV/PT 3.9.1.3 Interpretation of results. Sample genotype is determined by examining the melting curve for the presence or

absence of peaks whose T_m is specific for a wild-type or mutant allele.

The laboratory should establish acceptable T_m ranges for the wild-type and mutant alleles, as the T_m values have inter- and intrarun variability. In addition, it is useful to monitor and establish a range for the ΔT_m [T_m (wild type) – T_m (mutant)]. The ΔT_m is less variable than the T_m values themselves and is a more useful value to help identify additional sequence variations.

Fluorescent melting curve analysis allows the detection of additional sequence variations in the target sequence. These additional variations are identified by altered melting curve profiles that have peaks whose T_m does not match the wild-type or mutant allele. For example, the rare polymorphisms (G1689A and A1692C) result in peak shifts in a factor V Leiden (G1691A) assay. The peak shifts may be subtle (< 1°C). Sequence variations are most easily identified by a ΔT_m value that is outside the range for normal and mutant alleles. It is recommended that sequence variants be confirmed by DNA sequencing.

FV/PT 3.10 Liquid bead

FV/PT 3.10.1 Overview. Liquid bead arrays provide simple and high-throughput analysis of DNA polymorphisms with discrete detection of wild-type and mutant alleles in a complex genetic assay. Bead-array platforms use either universal tags or allele-specific capture probes that are covalently immobilized on spectrally distinct microspheres. Because microsphere sets can be distinguished by their spectral addresses, they can be combined, allowing as many as 100 analytes to be measured simultaneously in a single reaction vessel. A third fluorochrome coupled to a reporter molecule quantifies the molecular interaction that has occurred at the microsphere surface. The microspheres, or beads, are dyed internally with one or more fluorophores, the ratio of which can be combined to make multiple bead sets. Capture probes are covalently attached to beads via a terminal amine modification. Bead arrays offer significant advantages over other array technologies in that hybridization occurs rapidly in a single tube, the testing volume scales to a microtiter plate and unlike glass or membrane microarrays, bead solutions can be quality tested as individual components. Laboratories most easily use bead arrays in the context of validated ASRs because the reagents incorporate proprietary elements and none of the commercially available products are FDA cleared.^{37–40}

FV/PT 3.10.2 Multiplex PCR amplification. All general guidelines for multiplex PCR amplification apply to liquid bead array-based detection. All commercial products use a single multiplex PCR with proprietary primers designed to accommodate the hybridization and detection system being used. Because liquid bead arrays work well with various front-end chemistries, including oligo ligation, allele-specific single base extension, allele-specific oligonucleotide (ASO) hybridization, and allele-specific primer extension (ASPE), the detection chemistry of the particular detection

format can be incorporated into the PCR and/or subsequent amplicon modification steps.

FV/PT 3.10.3 Hybridization and detection. One commercial platform uses biotin-modified PCR products that are hybridized to allele-specific capture probes on different beads.^{37–39} Another uses allele-specific primer extensions of the PCR product such that “universal tags” are incorporated into the product for allele discrimination.⁴⁰ The biotinylated PCR product, or extended PCR product, is then hybridized to either capture probes or “universal antitags,” respectively, that are covalently bound to the beads. Both platforms use a reporter fluorophore, streptavidin-phycoerythrin, in or before the hybridization reaction. After hybridization, the modified amplicon is bound to a reporter substrate and transferred directly to a detection instrument without posthybridization purification. The sample genotype is assigned by comparing the relative hybridization signal between the wild-type and mutant alleles. The generation of electronic data facilitates the development of automated analysis software and database archiving. The reaction is analyzed for bead identity and associated hybridization signal intensity. Lasers interrogate hybridized microspheres individually as they pass single-file in a rapidly flowing stream. Thousands of microspheres are interrogated per second resulting in an analysis system capable of analyzing and reporting up to 100 different hybridization reactions in a single well of a 96-well plate in just a few seconds.

FV/PT 3.10.4 Visualization and interpretation of results. Output files generated during detection are automatically processed and made available in a report format through customized software. The software should allow for controlled access to data, patient reports, comments, and sample history. Electronic data output is archived into a database format for data integrity, quality control tracking, and result trending and incorporates batch processing of results, highlighting samples with mutations, and genotype calling.

FV/PT 3.10.5 Quality control and controls. It is not feasible to include a gDNA for each positive assay control in each run due to reagent cost and batch size limitations. However, quality control on a new lot of beads should include gDNA-based testing for each mutation. At a minimum, during routine testing, it is recommended that each run include at least one positive assay control and that all positive controls be tested on a rotating basis. The use of either genomic or synthetic compound heterozygotes can also minimize the number of positive controls. The last sample in each batch should be a no template control, to assess for reagent contamination by previous or current amplicons. The ratio of wild-type to mutant signal, adjusted for background for each control, should fall into previously set ranges that maximize the signal-to-noise ratio and the no template controls should fall below an arbitrary preset detection limit.

FV/PT 3.11 Other testing methodologies. Commercial kits and new methodologies for detecting the factor V Leiden and prothrombin 20210G>A mutations are being introduced fre-

quently. It is the responsibility of the laboratory and/or medical director of each laboratory to evaluate and validate any new methodology before it is utilized for clinical testing.

FV/PT 3.12 Laboratory result interpretations (postanalytical). The following elements must be included in the report, in addition to the items described in the general Standards and Guidelines for Clinical Genetics Laboratories.

FV/PT 3.12.1 Method used

FV/PT 3.12.2 Patient’s result. The patient’s result classified into the following categories, i.e., normal, heterozygous, or homozygous.

FV/PT 3.12.2.1 “Positive” results. All “positive” results, i.e., heterozygous or homozygous for the factor V Leiden mutation and/or the prothrombin 20210G>A mutation, should state that it is important for individuals possessing either one or both of these mutations to understand the clinical risks and the genetic implications of their result. Patients should be counseled by their physician or genetic counselor.

FV/PT 3.12.2.2 Comments on phenotype. Comments on phenotype, if included, should be abstract rather than case-specific. The following concepts apply.

FV/PT 3.12.2.2.1 Factor V Leiden heterozygote. Individuals heterozygous for the R506Q mutation have an approximately 4- to 7- or 8-fold increased risk of venous thrombosis as compared to individuals without the mutation.^{41,42}

FV/PT 3.12.2.2.2 Prothrombin 20210G>A heterozygote. Individuals heterozygous for the prothrombin 20210G>A mutation have an approximately 2- to 4-fold increased risk of venous thrombosis as compared to individuals without the mutation.⁸

FV/PT 3.12.2.2.3 Factor V Leiden homozygote. Individuals homozygous for the R506Q mutation have an approximately 80-fold increased risk of venous thrombosis as compared to individuals without the mutation.⁸

FV/PT 3.12.2.2.4 Prothrombin 20210G>A homozygote. The number of individuals reported to be homozygous for the prothrombin 20210G>A mutation is so small that it is difficult to determine the risk of venous thrombosis as compared to individuals without the mutation.²⁹

FV/PT 3.12.2.2.5 Factor V Leiden and Prothrombin 20210G>A compound heterozygote. Individuals carrying both the factor V Leiden and prothrombin 20210G>A mutations have a 20-fold more likely chance of having a venous thrombosis than individuals without either mutation.⁴³ Between 1% and 10% of symptomatic carriers of the factor V Leiden mutation also carry the prothrombin 20210G>A mutation. These individuals have a 50- to 80-fold relative risk of thrombosis as compared to homozygotes for the factor V Leiden mutation.^{10,44–49}

FP/PT 4 ALTERNATIVE TESTING METHODS

The phenotypic condition associated with factor V Leiden, APC resistance, can be diagnosed by functional coagulation testing. Depending upon the method used, the functional assay may pick up the cases of APC resistance not due to factor V Leiden. The current generation of functional assays can be used in patients on anticoagulant therapy.

There are also functional assays that measure overall rates of coagulation, but they cannot be used in patients on anticoagulant therapy and are not as robust as the DNA test.

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