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(54) Title: METHOD EVOLVED FOR RECOGNITION OF THROMBOPHILIA (MERT)

(57) Abstract: Methods for predicting an individual's genetic risk for developing venous thrombosis in diverse ethnic populations is disclosed, as are arrays and kits which can be used to practice the method. The method includes screening for mutations, polymorphisms, or both, in at least eight venous thrombosis-related molecules, such as antithrombin III, protein C, protein S, fibrinogen, factor V, prothrombin (factor II), methylenetetrahydrofolate reductase (MTHFR), and angiotensin I-converting enzyme (ACE) molecules which are associated with venous thrombosis.

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## METHOD EVOLVED FOR RECOGNITION OF THROMBOPHILIA (MERT)

### CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No.  
5 60/537,463 filed January 15, 2004, which is hereby incorporated by reference in its  
entirety.

### FIELD

This application relates to methods of predicting an individual's genetic  
10 susceptibility to venous thrombosis, as well as kits that can be used to practice the  
disclosed methods.

### BACKGROUND

Venous thrombosis affects 1 per 1000 individuals annually and is one of the  
15 leading causes of mortality and morbidity resulting in approximately 300,000  
hospitalizations and 50,000 fatalities per year in the United States alone (Rosendaal,  
*Thromb. Haemost.* 78:1-6, 1997; Nordstrom *et al.*, *J. Inter. Med.* 232:155-60, 1992;  
and Hansson *et al.*, *Arch. Intern. Med.* 157:1665-70, 1997).

Numerous conditions predispose an individual to venous thrombosis. Examples  
20 of such risk factors include pregnancy, puerperium, use of oral contraceptives and/or  
hormone replacement therapy, trauma, surgery, fractures, prolonged immobilization,  
advanced age, antiphospholipid antibodies, previous thrombosis history,  
myeloproliferative disorders, malignancy, and mild-to-moderate hyperhomocysteinemia  
(Abramson *et al.*, *Southern Med. J.* 94:1013-20, 2001; and Seligsohn and Lubetsky, *N.*  
25 *Engl. J. Med.* 344:1222-31, 2001). Venous thrombosis often occurs in the lower leg as  
a deep venous thrombosis (DVT) which often leads to pulmonary emboli that are often  
fatal. It is particularly unfortunate that such thromboembolic phenomena often occur in  
already physiologically compromised patients.

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In addition to acquired risk factors for venous thrombosis, a number of seemingly monogenic, autosomal dominant, variably penetrant genetic mutations or polymorphisms impart an increased risk for venous thrombosis. Examples of such mutations or polymorphisms are in genes that encode procoagulant proteins (Factor V, prothrombin and fibrinogen), natural anticoagulant proteins (protein C, protein S and antithrombin III) and other thrombosis related proteins (angiotensin-I converting enzyme and methylenetetrahydrofolate reductase). Therefore, venous thrombosis is a complex genetic disorder. Genetic defects leading to hyperactivity of the coagulation system are present in a large proportion of patients with venous thrombosis. More than 60% of the predisposition to thrombosis is attributable to genetic components (Souto *et al.*, *Am. J. Hum. Genet.* 67:1452-9, 2000).

Previous reports describe screening for one or more polymorphisms associated with thrombosis, for example by using PCR (Harrington *et al.*, *Clin. Chem. Lab. Med.* 41:496-500, 2003), or microplate array diagonal gel electrophoresis (Bauer *et al.*, *Thromb. Haemost.* 84:396-400, 2000). Although the use of microarray technology to screen for mutations in particular genes involved in venous thrombosis has been proposed, these microarrays are limited because they have a low predictive value and only detect mutations that are prevalent in Caucasian populations (for example see Pecheniuk *et al.*, *Blood Coagul. Fibrinolysis* 11:683-700, 2000; Pollak *et al.*, *Ital. Heart J.* 2:568-72, 2001; Evans and Lee-Tataseo, *Clin. Chem.* 48:1406-11, 2002; Schrijver *et al.*, *Am. J. Clin. Pathol.* 119:490-6, 2003; Erali *et al.*, *Clin. Chem.* 49:732-9, 2003). Others have indicated that microarray technology needs to undergo further development before it is available for screening the numerous genetic mutations and polymorphisms involved in thrombosis (Grody, *Annu. Rev. Med.*, 54:473-90, 2003).

Therefore, there is a need for a method that can accurately predict the risk of an individual for developing venous thrombosis, which can be used to screen multiple ethnic populations.

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**SUMMARY**

Although venous thrombosis is one of the leading causes of morbidity and mortality in developed countries, it is an avoidable disease by the use of prophylactic treatment with currently available anticoagulants such as unfractionated heparin, low  
5 molecular-weight heparins, aspirin, and coumadin/warfarin. Thus, it is beneficial to estimate the individual thrombotic risk to develop stratification protocols for an individual risk-adapted prophylaxis to avoid the development of venous thromboembolism. For this stratification, the individual risk associated with single or combined risk factors of hemostasis is estimated.

10 The inventors have identified combinations of mutations and polymorphisms in venous thrombosis-related molecules that allow one to predict the genetic susceptibility of an individual to developing venous thrombosis with high accuracy in several ethnic populations. In one example, the combinations of recurrent mutations and polymorphisms in molecules that are statistically associated with venous thrombosis  
15 allow for prediction of the overall genetic susceptibility of an individual to developing venous thrombosis with high accuracy in several ethnic populations. Recurrent mutations and polymorphisms are those that occur in more than one family, such as at least two genetically distinct families. For example, a mutation or polymorphism that is only observed in a one family is not a recurrent mutation or polymorphism.

20 For example, the disclosed statistical analysis regarding concurrent testing of at least ten venous thrombosis associated genetic variations using the disclosed method demonstrated that the prediction of venous thrombosis is as accurate as at least 99% in Caucasians, at least 85% in Asians, and at least 88% in African populations. The disclosed methods, herein termed method evolved for recognition of thrombophilia  
25 (MERT), provide a rapid and cost-effective assay that allows for concurrent genetic testing in molecules statistically associated with venous thrombosis susceptibility, for example antithrombin III, protein C, protein S, fibrinogen, factor V, prothrombin (factor II), methylenetetrahydrofolate reductase (MTHFR), and angiotensin I-converting enzyme (ACE).

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In one example, the method includes determining whether a subject has one or more mutations, polymorphisms, or both, in venous thrombosis-associated molecules that comprise, consist essentially of, or consist of, sequences from antithrombin III, protein C, protein S, fibrinogen, factor V, factor II, MTHFR and ACE. In one example, 5 asymptomatic individuals are screened before or during their exposure to high risk situations that provoke thrombosis, such as pregnancy, puerperium, use of oral contraceptives or hormone replacement therapy, previous thrombosis history, prolonged immobilization, myeloproliferative disorders, malignancy, surgery, bone fracture, advanced age, antiphospholipid antibodies, or combinations thereof.

10 Although there are some already existing tests for screening up to six thrombophilia susceptibility single nucleotide polymorphisms, they have limited potential and a maximum predictive value of 1.7%. Such tests have a screening capacity that only allows them to detect single nucleotide polymorphisms (SNPs) that are prevalent only in Caucasian populations (Erali *et al.*, *Clin. Chem.* 49:5, 2003; Evans 15 *et al.*, *Clin. Chem* 48:1406-11, 2002). In contrast, the methods and arrays disclosed herein are the first offering a highly accurate, overall venous thrombosis genetic susceptibility prediction, for example by screening mutations and polymorphisms (not only for SNPs but also for insertions and deletions) in those genes statistically associated with venous thrombosis. In particular examples, all known recurrent 20 mutations and polymorphisms statistically associated with venous thrombosis are screened, or a subset of all such known mutations and polymorphisms. In some examples, a mutation or and polymorphism is statistically associated with venous thrombosis if it has a p-value of less than 0.005.

In particular examples, the method uses genomic DNA microarray technology to 25 detect a subject's overall genetic susceptibility to venous thrombosis, and links the microarray data directly to the combined likelihood ratio for the panel of VT associated susceptibility genes applicable to diverse ethnic populations.

In a particular example, the method includes amplifying nucleic acid molecules obtained from a subject to obtain amplification products. The amplification products

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can comprise, consist essentially of, or consist of, sequences from antithrombin III, protein C, protein S, fibrinogen, factor V, prothrombin (factor II), methylenetetrahydrofolate reductase (MTHFR) and angiotensin I-converting enzyme (ACE) genes. The resulting amplification products are contacted with or applied to an array. The array includes oligonucleotide probes capable of hybridizing to antithrombin III, protein C, protein S, fibrinogen, factor V, factor II, MTHFR and ACE sequences that include one or more mutations, one or more polymorphisms, or combinations thereof. Examples of particular mutations and polymorphisms are provided in Table 1. In some examples, the array further includes oligonucleotides capable of hybridizing to wild-type antithrombin III, wild-type protein C, wild-type protein S, wild-type fibrinogen, wild-type factor V, wild-type factor II, wild-type MTHFR and wild-type ACE. The amplification products are incubated with the array for a time sufficient to allow hybridization between the amplification products and oligonucleotide probes, thereby forming amplification products:oligonucleotide probe complexes. The amplification products:oligonucleotide probe complexes are then analyzed to determine if the amplification products include one or more mutations, polymorphisms, or both, in antithrombin III, protein C, protein S, fibrinogen, factor V, factor II, MTHFR or ACE. The presence of one or more mutations or one or more polymorphisms indicates that the subject has a genetic predisposition for venous thrombosis. In particular examples, the presence of more than one mutation or polymorphism indicates that the subject is at a greater risk for venous thrombosis than is a subject having only one mutation or polymorphism.

The disclosed method can accurately assess the overall genetic risk of developing venous thrombosis and thereby lead to avoiding venous thrombosis, for example by initiating appropriate prophylactic therapies in appropriate circumstances. The results presented herein demonstrate that concurrent use of a panel of genetic tests for at least eight molecules associated with venous thrombosis increases the positive predictive value more than 30 fold, when used for detecting venous thrombosis or a predisposition to its development. Therefore, methods of selecting venous thrombosis

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therapy are disclosed, which include detecting a mutation, a polymorphism, or combinations thereof (such as one or more substitutions, deletions or insertions) in at least one VT-related molecule of a subject, using the methods disclosed herein and if such mutation or polymorphism is identified, selecting a treatment to avoid venous thrombosis, delay the onset of venous thrombosis, or minimize its consequences.

Also disclosed are arrays capable of rapid, cost-effective multiple genetic testing for venous thrombosis genetic susceptibility, such as overall venous thrombosis genetic susceptibility. Such arrays include oligonucleotides that are complementary to antithrombin III, protein C, protein S, factor V, factor II, fibrinogen, MTHFR and ACE wild-type or mutated sequences, or both. Kits including such arrays for detecting a genetic predisposition to venous thrombosis in a subject are also disclosed.

The foregoing and other features and advantages of the disclosure will become more apparent from the following detailed description of a several embodiments.

## SEQUENCE LISTING

The nucleic acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand.

### ATIII

SEQ ID NO: 1 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position 2770.

SEQ ID NO: 2 is an oligonucleotide sequence that can be used to probe for a 2770 insT in antithrombin III.

SEQ ID NO: 3 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide positions 5311-5320.

SEQ ID NO: 4 is an oligonucleotide sequence that can be used to probe for a 5311-5320 del6bp in antithrombin III.

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SEQ ID NO: 5 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide positions 5356-5364.

SEQ ID NO: 6 is an oligonucleotide sequence that can be used to probe for a 5356-5364, delCTT in antithrombin III.

5 SEQ ID NO: 7 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position 5381 C.

SEQ ID NO: 8 is an oligonucleotide sequence that can be used to probe for a 5381C/T replacement in antithrombin III.

10 SEQ ID NO: 9 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position 5390 C.

SEQ ID NO: 10 is an oligonucleotide sequence that can be used to probe for a 5390 C/T replacement in antithrombin III.

SEQ ID NO: 11 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position 5493 A.

15 SEQ ID NO: 12 is an oligonucleotide sequence that can be used to probe for a 5493 A/G replacement in antithrombin III.

SEQ ID NO: 13 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position  $\alpha(16)$ Arg: CGT6490 C.

20 SEQ ID NO: 14 is an oligonucleotide sequence that can be used to probe for a 6490 C/T replacement in antithrombin III.

SEQ ID NO: 15 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position  $\alpha(16)$ Arg: CGT9788 G.

SEQ ID NO: 16 is an oligonucleotide sequence that can be used to probe for a 9788 G/A replacement in antithrombin III.

25 SEQ ID NO: 17 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position  $\alpha(19)$ Arg: AGG9819 C.

SEQ ID NO: 18 is an oligonucleotide sequence that can be used to probe for a 9819 C/T replacement in antithrombin III.

SEQ ID NO: 19 is an oligonucleotide sequence that can be used to probe for a

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wild-type antithrombin III sequence at nucleotide position 13342.

SEQ ID NO: 20 is an oligonucleotide sequence that can be used to probe for a 13342 insA in antithrombin III.

5 SEQ ID NO: 21 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position 13380 T.

SEQ ID NO: 22 is an oligonucleotide sequence that can be used to probe for a 13380 T/C replacement in antithrombin III.

SEQ ID NO: 23 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position  $\beta(14)$ Arg, CGT6460 A.

10 SEQ ID NO: 24 is an oligonucleotide sequence that can be used to probe for a 6460 A/G replacement in antithrombin III.

SEQ ID NO: 25 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position  $\beta(68)$ Ala, GCT13262 G.

15 SEQ ID NO: 26 is an oligonucleotide sequence that can be used to probe for a 13262 G/A replacement in antithrombin III.

SEQ ID NO: 27 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position  $\beta(255)$ Arg, CGT13268 G.

SEQ ID NO: 28 is an oligonucleotide sequence that can be used to probe for a 13268 G/C replacement in antithrombin III.

20 SEQ ID NO: 29 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position  $\gamma(275)$ Arg, CGC13268 G.

SEQ ID NO: 30 is an oligonucleotide sequence that can be used to probe for a 13268 G/T replacement in antithrombin III.

25 SEQ ID NO: 31 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position  $\gamma(275)$ Arg, CG13295 C.

SEQ ID NO: 32 is an oligonucleotide sequence that can be used to probe for a 13295 C/T replacement in antithrombin III.

SEQ ID NO: 33 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position  $\gamma(292)$ Gly, GGC13296 G.

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SEQ ID NO: 34 is an oligonucleotide sequence that can be used to probe for a 13296 G/A replacement in antithrombin III.

SEQ ID NO: 35 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position  $\gamma(308)$ Asn, AAT13299 C.

5 SEQ ID NO: 36 is an oligonucleotide sequence that can be used to probe for a 13299 C/T replacement in antithrombin III.

SEQ ID NO: 37 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position  $\gamma(318)$ Asp, GAC2484 T.

10 SEQ ID NO: 38 is an oligonucleotide sequence that can be used to probe for a  $\gamma(318)$ Asp/Gly: GAC/GGC2484 T/A replacement in antithrombin III.

SEQ ID NO: 39 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position Thr312, ACT2586 C.

SEQ ID NO: 40 is an oligonucleotide sequence that can be used to probe for a 2586 C/T replacement in antithrombin III.

15 SEQ ID NO: 41 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position 2603 C.

SEQ ID NO: 42 is an oligonucleotide sequence that can be used to probe for a 2603 C/T replacement in antithrombin III.

20 SEQ ID NO: 43 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position 2604 G.

SEQ ID NO: 44 is an oligonucleotide sequence that can be used to probe for a 2604 G/A replacement in antithrombin III.

SEQ ID NO: 45 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position 2759 C.

25 SEQ ID NO: 46 is an oligonucleotide sequence that can be used to probe for a 2759 C/T replacement in antithrombin III.

SEQ ID NO: 47 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position 5382 G.

SEQ ID NO: 48 is an oligonucleotide sequence that can be used to probe for a

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5382 G/A replacement in antithrombin III.

SEQ ID NO: 49 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position 13324 C.

5 SEQ ID NO: 50 is an oligonucleotide sequence that can be used to probe for a 13324 C/A replacement in antithrombin III.

SEQ ID NO: 51 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position 13328 G.

SEQ ID NO: 52 is an oligonucleotide sequence that can be used to probe for a 13328 G/A replacement in antithrombin III.

10 SEQ ID NO: 53 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position 13333 C.

SEQ ID NO: 54 is an oligonucleotide sequence that can be used to probe for a 13333 C/G replacement in antithrombin III.

15 SEQ ID NO: 55 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position 13337 C.

SEQ ID NO: 56 is an oligonucleotide sequence that can be used to probe for a 13337 C/A replacement in antithrombin III.

SEQ ID NO: 57 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position 13338 C.

20 SEQ ID NO: 58 is an oligonucleotide sequence that can be used to probe for a 13338 C/T replacement in antithrombin III.

SEQ ID NO: 59 is an oligonucleotide sequence that can be used to probe for a wild-type antithrombin III sequence at nucleotide position 13392 G.

25 SEQ ID NO: 60 is an oligonucleotide sequence that can be used to probe for a 13392 G/C replacement in antithrombin III.

### **Protein C**

SEQ ID NO: 61 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 3363/3364 in protein C41G.

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SEQ ID NO: 62 is an oligonucleotide sequence that can be used to probe for a 41G/A replacement in protein C.

SEQ ID NO: 63 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 1357C.

5 SEQ ID NO: 64 is an oligonucleotide sequence that can be used to probe for a 1357 C/T replacement in protein C.

SEQ ID NO: 65 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 1381C.

10 SEQ ID NO: 66 is an oligonucleotide sequence that can be used to probe for a 1381 C/T replacement in protein C.

SEQ ID NO: 67 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 3103C.

SEQ ID NO: 68 is an oligonucleotide sequence that can be used to probe for a 3103 C/T replacement in protein C.

15 SEQ ID NO: 69 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 3169 T.

SEQ ID NO: 70 is an oligonucleotide sequence that can be used to probe for a 3169 T/C replacement in protein C.

20 SEQ ID NO: 71 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 3217 G.

SEQ ID NO: 72 is an oligonucleotide sequence that can be used to probe for a 3217 G/T replacement in protein C.

SEQ ID NO: 73 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 3222 G.

25 SEQ ID NO: 74 is an oligonucleotide sequence that can be used to probe for a 3222 G/A replacement in protein C.

SEQ ID NO: 75 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 3222 G.

SEQ ID NO: 76 is an oligonucleotide sequence that can be used to probe for a

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3222 G/T replacement in protein C.

SEQ ID NO: 77 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 3359G.

5 3359 G/A replacement in protein C.

SEQ ID NO: 79 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 3360 C.

SEQ ID NO: 80 is an oligonucleotide sequence that can be used to probe for a 3360 C/A replacement in protein C.

10 SEQ ID NO: 81 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 3363/3364 in protein C.

SEQ ID NO: 82 is an oligonucleotide sequence that can be used to probe for a 3363/4 insC in protein C.

15 SEQ ID NO: 83 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 3438 C.

SEQ ID NO: 84 is an oligonucleotide sequence that can be used to probe for a 3438 C/T replacement in protein C.

SEQ ID NO: 85 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 6128 T.

20 SEQ ID NO: 86 is an oligonucleotide sequence that can be used to probe for a 6128 T/C replacement in protein C.

SEQ ID NO: 87 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 6152 C.

25 SEQ ID NO: 88 is an oligonucleotide sequence that can be used to probe for a 6152 C/T replacement in protein C.

SEQ ID NO: 89 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 6182 C.

SEQ ID NO: 90 is an oligonucleotide sequence that can be used to probe for a 6182 C/T replacement in protein C.

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SEQ ID NO: 91 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 6216 C.

SEQ ID NO: 92 is an oligonucleotide sequence that can be used to probe for a 6216 C/T replacement in protein C.

5 SEQ ID NO: 93 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 6245 C.

SEQ ID NO: 94 is an oligonucleotide sequence that can be used to probe for a 6245 C/T replacement in protein C.

10 SEQ ID NO: 95 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 6246 G.

SEQ ID NO: 96 is an oligonucleotide sequence that can be used to probe for a 6246 G/A replacement in protein C.

SEQ ID NO: 97 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 6265 G.

15 SEQ ID NO: 98 is an oligonucleotide sequence that can be used to probe for a 6265 G/C replacement in protein C.

SEQ ID NO: 99 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 6274 C.

20 SEQ ID NO: 100 is an oligonucleotide sequence that can be used to probe for a 6274 C/T replacement in protein C.

SEQ ID NO: 101 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 7176 G.

SEQ ID NO: 102 is an oligonucleotide sequence that can be used to probe for a 7176 G/A replacement in protein C.

25 SEQ ID NO: 103 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 7253 C.

SEQ ID NO: 104 is an oligonucleotide sequence that can be used to probe for a 7253 C/T replacement in protein C.

SEQ ID NO: 105 is an oligonucleotide sequence that can be used to probe for a

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wild-type protein C sequence at nucleotide position 8403 C.

SEQ ID NO: 106 is an oligonucleotide sequence that can be used to probe for a 8403 C/T replacement in protein C.

5 SEQ ID NO: 107 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8481 A.

SEQ ID NO: 108 is an oligonucleotide sequence that can be used to probe for a 8481 A/G replacement in protein C.

SEQ ID NO: 109 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8485-7.

10 SEQ ID NO: 110 is an oligonucleotide sequence that can be used to probe for a 8485/6 delAC or 8486/7 delCA in protein C.

SEQ ID NO: 111 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8551 C.

15 SEQ ID NO: 112 is an oligonucleotide sequence that can be used to probe for a 8551 C/T replacement in protein C.

SEQ ID NO: 113 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8559 G.

SEQ ID NO: 114 is an oligonucleotide sequence that can be used to probe for a 8559 G/A replacement in protein C.

20 SEQ ID NO: 115 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8571 C.

SEQ ID NO: 116 is an oligonucleotide sequence that can be used to probe for a 8571 C/T replacement in protein C.

25 SEQ ID NO: 117 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8572 G.

SEQ ID NO: 118 is an oligonucleotide sequence that can be used to probe for a 8572 G/A replacement in protein C.

SEQ ID NO: 119 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8589 G.

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SEQ ID NO: 120 is an oligonucleotide sequence that can be used to probe for a 8589 G/A replacement in protein C.

SEQ ID NO: 121 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8604 G.

5 SEQ ID NO: 122 is an oligonucleotide sequence that can be used to probe for a 8604 G/A replacement in protein C.

SEQ ID NO: 123 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8608 C.

10 SEQ ID NO: 124 is an oligonucleotide sequence that can be used to probe for a 8608 C/T replacement in protein C.

SEQ ID NO: 125 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8631 C.

SEQ ID NO: 126 is an oligonucleotide sequence that can be used to probe for a 8631 C/T replacement in protein C.

15 SEQ ID NO: 127 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8678-80.

SEQ ID NO: 128 is an oligonucleotide sequence that can be used to probe for a 8678-80 del3nt in protein C.

20 SEQ ID NO: 129 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8689 T.

SEQ ID NO: 130 is an oligonucleotide sequence that can be used to probe for a 8689 T/C replacement in protein C.

SEQ ID NO: 131 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8695 C.

25 SEQ ID NO: 132 is an oligonucleotide sequence that can be used to probe for a 8695 C/T replacement in protein C.

SEQ ID NO: 133 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 84708763 G.

SEQ ID NO: 134 is an oligonucleotide sequence that can be used to probe for a

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8763 G/A replacement in protein C.

SEQ ID NO: 135 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8857.

5 SEQ ID NO: 136 is an oligonucleotide sequence that can be used to probe for a 8857 delG in protein C.

SEQ ID NO: 137 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8895 A.

SEQ ID NO: 138 is an oligonucleotide sequence that can be used to probe for a 8895 A/C replacement in protein C.

10 SEQ ID NO: 139 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8924 C.

SEQ ID NO: 140 is an oligonucleotide sequence that can be used to probe for a 8924 C/G replacement in protein C.

15 SEQ ID NO: 141 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 1387 C.

SEQ ID NO: 142 is an oligonucleotide sequence that can be used to probe for a 1387 C/T replacement in protein C.

SEQ ID NO: 143 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 1388 G.

20 SEQ ID NO: 144 is an oligonucleotide sequence that can be used to probe for a 1388 G/A replacement in protein C.

SEQ ID NO: 145 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 1432 C.

25 SEQ ID NO: 146 is an oligonucleotide sequence that can be used to probe for a 1432 C/T replacement in protein C.

SEQ ID NO: 147 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position -34 TG6218 C.

SEQ ID NO: 148 is an oligonucleotide sequence that can be used to probe for a -34, delG6218 C/T replacement in protein C.

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SEQ ID NO: 149 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at -24 GTG (where 24 is the codon nucleotide position) 6219 G.

5 SEQ ID NO: 150 is an oligonucleotide sequence that can be used to probe for a 6219 G/A replacement in protein C.

SEQ ID NO: 151 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 7219 C.

SEQ ID NO: 152 is an oligonucleotide sequence that can be used to probe for a 7219 C/A replacement in protein C.

10 SEQ ID NO: 153 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8470 G.

SEQ ID NO: 154 is an oligonucleotide sequence that can be used to probe for a 8470 G/A replacement in protein C.

15 SEQ ID NO: 155 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8744 G.

SEQ ID NO: 156 is an oligonucleotide sequence that can be used to probe for a 8744 G/A replacement in protein C.

SEQ ID NO: 157 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8769 C.

20 SEQ ID NO: 158 is an oligonucleotide sequence that can be used to probe for a 8769 C/T replacement in protein C.

SEQ ID NO: 159 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8790 G.

25 SEQ ID NO: 160 is an oligonucleotide sequence that can be used to probe for a 8790 G/A replacement in protein C.

SEQ ID NO: 161 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position 8886 G.

SEQ ID NO: 162 is an oligonucleotide sequence that can be used to probe for a 8886 G/A replacement in protein C.

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SEQ ID NO: 163 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position -1654 C.

SEQ ID NO: 164 is an oligonucleotide sequence that can be used to probe for a -1654 C/T replacement in protein C.

5 SEQ ID NO: 165 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position -1641 A.

SEQ ID NO: 166 is an oligonucleotide sequence that can be used to probe for a -1641 A/G replacement in protein C.

10 SEQ ID NO: 167 is an oligonucleotide sequence that can be used to probe for a wild-type protein C sequence at nucleotide position -1476 A.

SEQ ID NO: 168 is an oligonucleotide sequence that can be used to probe for a -1476 A/T replacement in protein C.

### **Protein S**

15 SEQ ID NO: 169 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position -34, TGC.

SEQ ID NO: 170 is an oligonucleotide sequence that can be used to probe for a -34, delG in protein S.

20 SEQ ID NO: 171 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position -24, GTG.

SEQ ID NO: 172 is an oligonucleotide sequence that can be used to probe for a -24, GTG/GAG replacement in protein S.

SEQ ID NO: 173 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 19, GAA.

25 SEQ ID NO: 174 is an oligonucleotide sequence that can be used to probe for a 19, GAA/TAA replacement in protein S.

SEQ ID NO: 175 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 26, GAA.

SEQ ID NO: 176 is an oligonucleotide sequence that can be used to probe for a

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26, GAA/GCA replacement in protein S.

SEQ ID NO: 177 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 44.

5 44, delCTTA in protein S.

SEQ ID NO: 179 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 46, GTT.

SEQ ID NO: 180 is an oligonucleotide sequence that can be used to probe for a 46, GTT/CTT replacement in protein S.

10 SEQ ID NO: 181 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position intron d, exon 4, +1.

SEQ ID NO: 182 is an oligonucleotide sequence that can be used to probe for a intron d, G/A, exon 4, +1 replacement in protein S.

15 SEQ ID NO: 183 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 155, AAG.

SEQ ID NO: 184 is an oligonucleotide sequence that can be used to probe for a 155, AAG/GAG replacement in protein S.

SEQ ID NO: 185 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 217, AAT.

20 SEQ ID NO: 186 is an oligonucleotide sequence that can be used to probe for a 217, AAT/AGT replacement in protein S.

SEQ ID NO: 187 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 238, CAG.

25 238, CAG/TAG replacement in protein S.

SEQ ID NO: 189 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 265.

SEQ ID NO: 190 is an oligonucleotide sequence that can be used to probe for a 265, insT in protein S.

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SEQ ID NO: 191 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 293, TCA.

SEQ ID NO: 192 is an oligonucleotide sequence that can be used to probe for a 293, TCA/TGA replacement in protein S.

5 SEQ ID NO: 193 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 295, GGC.

SEQ ID NO: 194 is an oligonucleotide sequence that can be used to probe for a 295, GGC/GTC replacement in protein S.

10 SEQ ID NO: 195 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position intron j, exon 10, +5.

SEQ ID NO: 196 is an oligonucleotide sequence that can be used to probe for a intron j, G/A, exon 10, +5 replacement in protein S.

SEQ ID NO: 197 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 349, GAA.

15 SEQ ID NO: 198 is an oligonucleotide sequence that can be used to probe for a 349, GAA/AAA replacement in protein S.

SEQ ID NO: 199 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 372.

20 SEQ ID NO: 200 is an oligonucleotide sequence that can be used to probe for a 372, delCTTTTT, insAA in protein S.

SEQ ID NO: 201 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position intron k, exon 12, -9.

SEQ ID NO: 202 is an oligonucleotide sequence that can be used to probe for a intron k, A/G, exon 12, -9 replacement in protein S.

25 SEQ ID NO: 203 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position -25405, CTA.

SEQ ID NO: 204 is an oligonucleotide sequence that can be used to probe for a 405, CTA/CCA replacement in protein S.

SEQ ID NO: 205 is an oligonucleotide sequence that can be used to probe for a

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wild-type protein S sequence at position 410, CGA.

SEQ ID NO: 206 is an oligonucleotide sequence that can be used to probe for a 410, CGA/TGA replacement in protein S.

5 SEQ ID NO: 207 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 431.

SEQ ID NO: 208 is an oligonucleotide sequence that can be used to probe for a 431, insA in protein S.

SEQ ID NO: 209 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 465, TGG.

10 SEQ ID NO: 210 is an oligonucleotide sequence that can be used to probe for a 465, TGG/TGA replacement in protein S.

SEQ ID NO: 211 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 474, CGT.

15 SEQ ID NO: 212 is an oligonucleotide sequence that can be used to probe for a 474, CGT/TGT replacement in protein S.

SEQ ID NO: 213 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position intron b, exon 2 +5522, CAG.

SEQ ID NO: 214 is an oligonucleotide sequence that can be used to probe for a 522, CAG/TAG replacement in protein S.

20 SEQ ID NO: 215 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 534, CTG.

SEQ ID NO: 216 is an oligonucleotide sequence that can be used to probe for a 534, CTG/CGG replacement in protein S.

25 SEQ ID NO: 217 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position intron k, exon 11 +54625, TGT.

SEQ ID NO: 218 is an oligonucleotide sequence that can be used to probe for a 625, TGT/CGT replacement in protein S.

SEQ ID NO: 219 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position -2, CGT.

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SEQ ID NO: 220 is an oligonucleotide sequence that can be used to probe for a -  
2, CGT/CTT replacement in protein S.

SEQ ID NO: 221 is an oligonucleotide sequence that can be used to probe for a  
wild-type protein S sequence at position 9, AAA.

5 SEQ ID NO: 222 is an oligonucleotide sequence that can be used to probe for a  
9, AAA/GAA replacement in protein S.

SEQ ID NO: 223 is an oligonucleotide sequence that can be used to probe for a  
wild-type protein S sequence at position intron e, exon 5, +5.

10 SEQ ID NO: 224 is an oligonucleotide sequence that can be used to probe for a  
intron e, G/A, exon 5, +5 replacement in protein S.

SEQ ID NO: 225 is an oligonucleotide sequence that can be used to probe for a  
wild-type protein S sequence at position exon 15, 520 nt after the stop codon-25.

SEQ ID NO: 226 is an oligonucleotide sequence that can be used to probe for a -  
25, insT in protein S.

15 SEQ ID NO: 227 is an oligonucleotide sequence that can be used to probe for a  
wild-type protein S sequence at position 467, GTA.

SEQ ID NO: 228 is an oligonucleotide sequence that can be used to probe for a  
467, GTA/GGA replacement in protein S.

20 SEQ ID NO: 229 is an oligonucleotide sequence that can be used to probe for a  
wild-type protein S sequence at position 633.

SEQ ID NO: 230 is an oligonucleotide sequence that can be used to probe for a  
633, delAA in protein S.

SEQ ID NO: 231 is an oligonucleotide sequence that can be used to probe for a  
wild-type protein S sequence at position 636, TAA.

25 SEQ ID NO: 232 is an oligonucleotide sequence that can be used to probe for a  
636, TAA/TAT replacement in protein S.

SEQ ID NO: 233 is an oligonucleotide sequence that can be used to probe for a  
wild-type protein S sequence at position intron k, exon 11 +54.

SEQ ID NO: 234 is an oligonucleotide sequence that can be used to probe for a

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intron k, C/T, exon 11 +54 replacement in protein S.

SEQ ID NO: 235 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 460, TCC.

5 SEQ ID NO: 236 is an oligonucleotide sequence that can be used to probe for a 460, TCC/CCC replacement in protein S.

SEQ ID NO: 237 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position 626, CCA.

SEQ ID NO: 238 is an oligonucleotide sequence that can be used to probe for a 626, CCA/CCG replacement in protein S.

10 SEQ ID NO: 239 is an oligonucleotide sequence that can be used to probe for a wild-type protein S sequence at position exon 15, 520 nt after the stop codon.

SEQ ID NO: 240 is an oligonucleotide sequence that can be used to probe for a exon 15, C/A 520 nt after the stop codon replacement in protein S.

15 **Fibrinogen**

SEQ ID NO: 241 is an oligonucleotide sequence that can be used to probe for a wild-type fibrinogen sequence at position  $\alpha(16)$ Arg, CGT.

SEQ ID NO: 242 is an oligonucleotide sequence that can be used to probe for a  $\alpha(16)$ Arg/Cys: CGT/TGT replacement in fibrinogen.

20 SEQ ID NO: 243 is an oligonucleotide sequence that can be used to probe for a wild-type fibrinogen sequence at position  $\alpha(16)$ Arg, CGT.

SEQ ID NO: 244 is an oligonucleotide sequence that can be used to probe for a  $\alpha(16)$ Arg/His: CGT/CAT replacement in fibrinogen.

25 SEQ ID NO: 245 is an oligonucleotide sequence that can be used to probe for a wild-type fibrinogen sequence at position  $\alpha(19)$ Arg, AGG.

SEQ ID NO: 246 is an oligonucleotide sequence that can be used to probe for a  $\alpha(19)$ Arg/Gly: AGG/GGG replacement in fibrinogen.

SEQ ID NO: 247 is an oligonucleotide sequence that can be used to probe for a wild-type fibrinogen sequence at position  $\alpha(461)$ Lys, AAA.

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SEQ ID NO: 248 is an oligonucleotide sequence that can be used to probe for a  $\alpha(461)$ Lys/Stop: AAA/TAA replacement in fibrinogen.

SEQ ID NO: 249 is an oligonucleotide sequence that can be used to probe for a wild-type fibrinogen sequence at position  $\alpha(554)$ Arg, CGT.

5 SEQ ID NO: 250 is an oligonucleotide sequence that can be used to probe for a  $\alpha(554)$ Arg/Cys: CGT/TGT replacement in fibrinogen.

SEQ ID NO: 251 is an oligonucleotide sequence that can be used to probe for a wild-type fibrinogen sequence at position  $\beta(14)$ Arg , CGT.

10 SEQ ID NO: 252 is an oligonucleotide sequence that can be used to probe for a  $\beta(14)$ Arg/Cys: CGT/TGT replacement in fibrinogen.

SEQ ID NO: 253 is an oligonucleotide sequence that can be used to probe for a wild-type fibrinogen sequence at position  $\beta(68)$ Ala , GCT.

SEQ ID NO: 254 is an oligonucleotide sequence that can be used to probe for a  $\beta(68)$ Ala/Thr: GCT/ACT replacement in fibrinogen.

15 SEQ ID NO: 255 is an oligonucleotide sequence that can be used to probe for a wild-type fibrinogen sequence at position  $\beta(255)$ Arg , CGT.

SEQ ID NO: 256 is an oligonucleotide sequence that can be used to probe for a  $\beta(255)$ Arg/Cys: CGT/TGT replacement in fibrinogen.

20 SEQ ID NO: 257 is an oligonucleotide sequence that can be used to probe for a wild-type fibrinogen sequence at position  $\gamma(275)$ Arg, CGC.

SEQ ID NO: 258 is an oligonucleotide sequence that can be used to probe for a  $\gamma(275)$ Arg/Cys: CGC/TGC replacement in fibrinogen.

SEQ ID NO: 259 is an oligonucleotide sequence that can be used to probe for a wild-type fibrinogen sequence at position  $\gamma(275)$ Arg , CGC.

25 SEQ ID NO: 260 is an oligonucleotide sequence that can be used to probe for a  $\gamma(275)$ Arg/His: CGC/CAC replacement in fibrinogen.

SEQ ID NO: 261 is an oligonucleotide sequence that can be used to probe for a wild-type fibrinogen sequence at position  $\gamma(292)$ Gly , GGC.

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SEQ ID NO: 262 is an oligonucleotide sequence that can be used to probe for a  $\gamma(292)$ Gly/Val: GGC/GTC replacement in fibrinogen.

SEQ ID NO: 263 is an oligonucleotide sequence that can be used to probe for a wild-type fibrinogen sequence at position  $\gamma(308)$ Asn, AAT.

5 SEQ ID NO: 264 is an oligonucleotide sequence that can be used to probe for a  $\gamma(308)$ Asn/Lys: AAT/AAG replacement in fibrinogen.

SEQ ID NO: 265 is an oligonucleotide sequence that can be used to probe for a wild-type fibrinogen sequence at position  $\gamma(318)$ Asp, GAC.

10 SEQ ID NO: 266 is an oligonucleotide sequence that can be used to probe for a  $\gamma(318)$ Asp/Gly: GAC/GGC replacement in fibrinogen.

SEQ ID NO: 267 is an oligonucleotide sequence that can be used to probe for a wild-type fibrinogen sequence at position Thr312, ACT.

SEQ ID NO: 268 is an oligonucleotide sequence that can be used to probe for a Thr312Ala: ACT/GCT replacement in fibrinogen.

15

### **Factor V**

SEQ ID NO: 269 is an oligonucleotide sequence that can be used to probe for a wild-type factor V sequence at nucleotide position 1691G.

20 SEQ ID NO: 270 is an oligonucleotide sequence that can be used to probe for a 1691G/A replacement in factor V.

SEQ ID NO: 271 is an oligonucleotide sequence that can be used to probe for a wild-type factor V sequence at nucleotide position 1628G.

SEQ ID NO: 272 is an oligonucleotide sequence that can be used to probe for a 1628G/A replacement in factor V.

25 SEQ ID NO: 273 is an oligonucleotide sequence that can be used to probe for a wild-type factor V sequence at nucleotide position 4070A.

SEQ ID NO: 274 is an oligonucleotide sequence that can be used to probe for a 5382 G replacement in factor V.

SEQ ID NO: 275 is an oligonucleotide sequence that can be used to probe for a

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wild-type factor V sequence at nucleotide position 1090A.

SEQ ID NO: 276 is an oligonucleotide sequence that can be used to probe for a 1090A/G replacement in factor V.

5 SEQ ID NO: 277 is an oligonucleotide sequence that can be used to probe for a wild-type factor V sequence at nucleotide position 1091G.

SEQ ID NO: 278 is an oligonucleotide sequence that can be used to probe for a 1091G/C replacement in factor V.

### **Factor II**

10 SEQ ID NO: 279 is an oligonucleotide sequence that can be used to probe for a wild-type prothrombin sequence at nucleotide position 20210G.

SEQ ID NO: 280 is an oligonucleotide sequence that can be used to probe for a 20210G/A replacement in prothrombin.

### **MTHFR**

15 SEQ ID NO: 281 is an oligonucleotide sequence that can be used to probe for a wild-type MTHFR sequence at nucleotide position 677 C.

SEQ ID NO: 282 is an oligonucleotide sequence that can be used to probe for a 677 C/T replacement in MTHFR.

20 SEQ ID NO: 283 is an oligonucleotide sequence that can be used to probe for a wild-type MTHFR sequence at nucleotide position 1298 A.

SEQ ID NO: 284 is an oligonucleotide sequence that can be used to probe for a 1298 A/C replacement in MTHFR.

### **ACE**

25 SEQ ID NO: 285 is an oligonucleotide sequence that can be used to probe for the beginning portion of the wild-type ACE sequence with 288 bp insertion in intron 16.

SEQ ID NO: 286 is an oligonucleotide sequence that can be used to probe for the middle portion of the wild-type ACE sequence with 288 bp insertion in intron 16.

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SEQ ID NO: 287 is an oligonucleotide sequence that can be used to probe for a mutant type ACE sequence with 288 bp deletion in intron 16.

## DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

5

### Abbreviations and Terms

The following explanations of terms and methods are provided to better describe the present disclosure and to guide those of ordinary skill in the art in the practice of the present disclosure. The singular forms “a,” “an,” and “the” refer to one or more than one, unless the context clearly dictates otherwise. For example, the term “comprising a nucleic acid” includes single or plural nucleic acids and is considered equivalent to the phrase “comprising at least one nucleic acid.” The term “or” refers to a single element of stated alternative elements or a combination of two or more elements, unless the context clearly indicates otherwise. As used herein, “comprises” means “includes.” Thus, “comprising A or B,” means “including A, B, or A and B,” without excluding additional elements. For example, the phrase “mutations or polymorphisms” or “one or more mutations or polymorphisms” means a mutation, a polymorphism, or combinations thereof, wherein “a” can refer to more than one.

Unless explained otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. The materials, methods, and examples are illustrative only and not intended to be limiting.

**African:** A human racial classification that includes persons having origins in any of the black racial groups of Africa. In some examples, includes dark-skinned persons who are natives or inhabitants of Africa, as well as persons of African descent, such as African-Americans, wherein such persons also retain substantial genetic similarity to natives or inhabitants of Africa. In a particular example, an African is at least 1/64 African.

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**Amplifying a nucleic acid molecule:** To increase the number of copies of a nucleic acid molecule, such as a gene or fragment of a gene, for example a region of a venous thrombosis (VT)-associated gene. The resulting amplified products are called amplification products.

5 An example of *in vitro* amplification is the polymerase chain reaction (PCR), in which a biological sample obtained from a subject is contacted with a pair of oligonucleotide primers, under conditions that allow for hybridization of the primers to a nucleic acid molecule in the sample. The primers are extended under suitable conditions, dissociated from the template, and then re-annealed, extended, and  
10 dissociated to amplify the number of copies of the nucleic acid molecule. Other examples of *in vitro* amplification techniques include quantitative real-time PCR, strand displacement amplification (see USPN 5,744,311); transcription-free isothermal amplification (see USPN 6,033,881); repair chain reaction amplification (see WO 90/01069); ligase chain reaction amplification (see EP-A-320 308); gap filling ligase  
15 chain reaction amplification (see USPN 5,427,930); coupled ligase detection and PCR (see USPN 6,027,889); and NASBA™ RNA transcription-free amplification (see USPN 6,025,134).

**Angiotensin I-converting enzyme (ACE):** An enzyme that converts angiotensin I into the vasoconstrictor angiotensin II, and is involved in the degradation  
20 of bradykinin. Includes any ACE gene, cDNA, RNA, or protein from any organism, such as a human. Examples include the sequence disclosed in GenBank Accession No. BC048144 (as well as the corresponding genomic and protein sequence).

At least one variation in human ACE is associated with venous thrombosis: a polymorphism consisting of an insertion (ins) or deletion (del) of a 288-bp fragment in  
25 intron 16.

**Anticoagulants:** Agents that decrease or prevent abnormal blood clotting. Anticoagulants can avoid the formation of new clots, and prevent existing clots from growing (extending), for example by decreasing or stopping the production of proteins necessary for blood to clot. Examples include, but are not limited to, aspirin, heparin,

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and warfarin.

**Antithrombin III (AT III):** A member of the serpin (serine proteinase inhibitor) superfamily of proteins. AT III is the principal thrombin inhibitor, and has inhibitory effects on other coagulation factors, such as factors IXa, Xa, XIa and XIIa. In addition, AT III accelerates the dissociation of the factor VIIa-tissue factor complex and prevents its rebinding. Includes the product of any AT III gene, cDNA, or RNA, or an AT III protein from any organism, such as a human. Examples include the mRNA sequence disclosed in GenBank Accession No. NM\_000488 (as well as the corresponding genomic and protein sequence). The gene coding for human AT III is localized on chromosome 1q23-25, spans 13.4 kb of DNA and has seven exons.

Heterozygous AT III deficiency is associated with increased risk for venous thrombosis. The molecular basis of AT III deficiency is highly heterogeneous. AT III deficiency is divided into type I (low plasma levels of both functional and immunological AT III) and type II (variant AT III in plasma). Type II is further subdivided into RS (defective reactive site), HBS (defective heparin-binding site) and PE (pleiotropic, that is, multiple effects on function).

There are at least 127 distinct defects associated with AT III deficiency: 92 mutations for type I AT III deficiency (40 point mutations, 40 small insertions or deletions and 12 large deletions) and 35 mutations for type II AT III deficiency (12 RS, 12 HBS and 11 PE mutations, all point mutations). Among the type I mutations, at least 11 distinct mutations (7 point mutations and 4 deletions or insertions) have been described in multiple unrelated kindreds and the remaining mutations have been unique to single families which makes them individual mutations. In type II, 19 of the 35 mutations (seven RS, six HBS and six PE mutations) have been described in multiple unrelated kindreds and the remaining have been reported to be individual mutations.

Exemplary recurrent AT III gene mutations related to venous thrombosis are shown in Table 1.

**Array:** An arrangement of molecules, such as biological macromolecules (such as polypeptides or nucleic acids) or biological samples (such as tissue sections), in

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addressable locations on or in a substrate. A "microarray" is an array that is miniaturized so as to require or be aided by microscopic examination for evaluation or analysis. Arrays are sometimes called DNA chips or biochips.

The array of molecules ("features") makes it possible to carry out a very large number of analyses on a sample at one time. In certain example arrays, one or more molecules (such as an oligonucleotide probe) will occur on the array a plurality of times (such as twice), for instance to provide internal controls. The number of addressable locations on the array can vary, for example from a few (such as three) to at least 50, at least 100, at least 200, at least 250, at least 300, at least 500, at least 600, at least 1000, at least 10,000, or more. In particular examples, an array includes nucleic acid molecules, such as oligonucleotide sequences that are at least 15 nucleotides in length, such as about 15-40 nucleotides in length, such as at least 18 nucleotides in length, at least 21 nucleotides in length, or even at least 25 nucleotides in length. In one example, the molecule includes oligonucleotides attached to the array via their 5'- or 3'-end.

In particular examples, an array includes SEQ ID NOS: 1-287, or subsets thereof, such as odd-numbered SEQ ID NOS: 1-285 and SEQ ID NO: 286 (to detect wild-type VT-associated sequences), or even-numbered SEQ ID NOS: 2-284 and SEQ ID NO: 287 (to detect mutant or polymorphic VT-associated sequences), as well as at least 20 of the sequences shown in SEQ ID NOS: 1-287, such as at least 50, at least 75, at least 100, at least 150, at least 200, at least 250, or at least 260 of the sequences shown in SEQ ID NOS: 1-287.

Within an array, each arrayed sample is addressable, in that its location can be reliably and consistently determined within the at least two dimensions of the array. The feature application location on an array can assume different shapes. For example, the array can be regular (such as arranged in uniform rows and columns) or irregular. Thus, in ordered arrays the location of each sample is assigned to the sample at the time when it is applied to the array, and a key may be provided in order to correlate each location with the appropriate target or feature position. Often, ordered arrays are arranged in a symmetrical grid pattern, but samples could be arranged in other patterns

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(such as in radially distributed lines, spiral lines, or ordered clusters). Addressable arrays usually are computer readable, in that a computer can be programmed to correlate a particular address on the array with information about the sample at that position (such as hybridization or binding data, including for instance signal intensity). In some  
5 examples of computer readable formats, the individual features in the array are arranged regularly, for instance in a Cartesian grid pattern, which can be correlated to address information by a computer.

Also contemplated herein are protein-based arrays, where the probe molecules are or include proteins, or where the target molecules are or include proteins, and arrays  
10 including nucleic acids to which proteins/peptides are bound, or vice versa.

**Asian:** A human racial classification that includes persons having origins in any of the original peoples of the Far East, Southeast Asia, the Indian subcontinent, or the Pacific Islands. This area includes, for example, China, India, Japan, Korea, the Philippine Islands, and Samoa. In particular examples, Asians include persons of Asian  
15 descent, such as Asian-Americans, that retain substantial genetic similarity to natives or inhabitants of Asia. In a particular example, an Asian is at least 1/64 Asian.

**Binding or stable binding:** An association between two substances or molecules, such as the hybridization of one nucleic acid molecule to another (or itself) and the association of an antibody with a peptide. An oligonucleotide molecule binds or  
20 stably binds to a target nucleic acid molecule if a sufficient amount of the oligonucleotide molecule forms base pairs or is hybridized to its target nucleic acid molecule, to permit detection of that binding. Binding can be detected by any procedure known to one skilled in the art, such as by physical or functional properties of the target:oligonucleotide complex. For example, binding can be detected functionally by  
25 determining whether binding has an observable effect upon a biosynthetic process such as expression of a gene, DNA replication, transcription, translation, and the like.

Physical methods of detecting the binding of complementary strands of nucleic acid molecules, include but are not limited to, such methods as DNase I or chemical footprinting, gel shift and affinity cleavage assays, Northern blotting, dot blotting and

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light absorption detection procedures. For example, one method involves observing a change in light absorption of a solution containing an oligonucleotide (or an analog) and a target nucleic acid at 220 to 300 nm as the temperature is slowly increased. If the oligonucleotide or analog has bound to its target, there is a sudden increase in  
5 absorption at a characteristic temperature as the oligonucleotide (or analog) and target disassociate from each other, or melt. In another example, the method involves detecting a signal, such as a detectable label, present on one or both complementary strands.

The binding between an oligomer and its target nucleic acid is frequently  
10 characterized by the temperature ( $T_m$ ) at which 50% of the oligomer is melted from its target. A higher ( $T_m$ ) means a stronger or more stable complex relative to a complex with a lower ( $T_m$ ).

**Caucasian:** A human racial classification traditionally distinguished by physical characteristics such as very light to brown skin pigmentation and straight to wavy or  
15 curly hair, which includes persons having origins in any of the original peoples of Europe, North Africa, or the Middle East. Popularly, the word "white" is used synonymously with "Caucasian" in North America. Such persons also retain substantial genetic similarity to natives or inhabitants of Europe, North Africa, or the Middle East. In a particular example, a Caucasian is at least 1/64 Caucasian.

20 **cDNA (complementary DNA):** A piece of DNA lacking internal, non-coding segments (introns) and regulatory sequences which determine transcription. cDNA can be synthesized by reverse transcription from messenger RNA extracted from cells.

**Complementarity and percentage complementarity:** Molecules with complementary nucleic acids form a stable duplex or triplex when the strands bind,  
25 (hybridize), to each other by forming Watson-Crick, Hoogsteen or reverse Hoogsteen base pairs. Stable binding occurs when an oligonucleotide molecule remains detectably bound to a target nucleic acid sequence under the required conditions.

Complementarity is the degree to which bases in one nucleic acid strand base pair with the bases in a second nucleic acid strand. Complementarity is conveniently

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described by percentage, that is, the proportion of nucleotides that form base pairs between two strands or within a specific region or domain of two strands. For example, if 10 nucleotides of a 15-nucleotide oligonucleotide form base pairs with a targeted region of a DNA molecule, that oligonucleotide is said to have 66.67% complementarity  
5 to the region of DNA targeted.

In the present disclosure, "sufficient complementarity" means that a sufficient number of base pairs exist between an oligonucleotide molecule and a target nucleic acid sequence (such as antithrombin III, protein C, protein S, fibrinogen, factor V, factor II, MTHFR, and ACE) to achieve detectable binding. When expressed or measured by  
10 percentage of base pairs formed, the percentage complementarity that fulfills this goal can range from as little as about 50% complementarity to full (100%) complementary. In general, sufficient complementarity is at least about 50%, for example at least about 75% complementarity, at least about 90% complementarity, at least about 95% complementarity, at least about 98% complementarity, or even at least about 100%  
15 complementarity.

A thorough treatment of the qualitative and quantitative considerations involved in establishing binding conditions that allow one skilled in the art to design appropriate oligonucleotides for use under the desired conditions is provided by Beltz *et al. Methods Enzymol* 100:266-285, 1983, and by Sambrook *et al. (ed.), Molecular Cloning: A  
20 Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

**DNA (deoxyribonucleic acid):** A long chain polymer which includes the genetic material of most living organisms (some viruses have genes including ribonucleic acid, RNA). The repeating units in DNA polymers are four different  
25 nucleotides, each of which includes one of the four bases, adenine, guanine, cytosine and thymine bound to a deoxyribose sugar to which a phosphate group is attached. Triplets of nucleotides, referred to as codons, in DNA molecules code for amino acid in a polypeptide. The term codon is also used for the corresponding (and complementary) sequences of three nucleotides in the mRNA into which the DNA sequence is

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transcribed.

**Deletion:** The removal of one or more nucleotides from a nucleic acid sequence (or one or more amino acids from a protein sequence), the regions on either side of the removed sequence being joined together.

5           **Factor V (FV):** A protein that can act as a cofactor in the conversion of prothrombin to thrombin by factor Xa and  $\alpha$ -thrombin. Includes the product of any FV gene, cDNA, or RNA, or a FV protein from any organism, such as a human. Examples of FV nucleic acid sequences include the mRNA sequence disclosed in GenBank  
10           Accession No. NM\_000130 (as well as the corresponding genomic and protein sequence).

          FV circulates in the plasma as a 330-kDa single chain glycoprotein. Downregulation of the procoagulant activity of activated FV (FVa) is accomplished by activated protein C (APC)-mediated proteolysis of FVa at three different sequential cleavage sites. Factor V is first cleaved at Arg 506, then at Arg 306, and finally at Arg  
15           679. The cleavage of the peptide bond at Arg 506 is needed for the subsequent optimal exposure of cleavage sites at Arg 306 and Arg 679. Peptide bond cleavage at Arg 306 accounts for the initial 70% loss of activity and the subsequent cleavage at Arg 679 is responsible for the loss of the remaining activity.

          At least five single nucleotide substitutions in the human FV gene are associated  
20           with increased thrombosis risk: 1691G→A transition that results in a Arg506Gln polymorphism; 1628 G → A transition that results in a R485K polymorphism; 1091 G → C transition that results in a Arg306Thr mutation; 1090 A → G transition that results in a Arg306Gly mutation; and 4070 A → G transition that results in a His1299Arg polymorphism.

25           **Fibrinogen:** A plasma protein with multiple functions in blood clotting, such as fibrin clot formation, factor XIII-mediated fibrin crosslinking, nonsubstrate thrombin binding, platelet aggregation, and fibrinolysis. Includes the product of any fibrinogen gene, cDNA, RNA, or a fibrinogen protein from any organism, such as a human. Examples of fibrinogen nucleic acid sequences include the mRNA sequences disclosed

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in GenBank Accession Nos. NM\_021871.1, BC007030, and NM\_021870 (for the  $\alpha$ ,  $\beta$ , and  $\gamma$ ) subunits respectively, as well as the corresponding genomic and protein sequences).

Human fibrinogen is a 340-kDa glycoprotein, composed of two identical  
5 subunits linked through a disulfide bond. Each subunit includes three polypeptide chains ( $\alpha$ ,  $\beta$ , and  $\gamma$ ), which are encoded by three separate genes on the long arm of human chromosome 4. Dysfibrinogenemia is caused by a variety of structural abnormalities in the fibrinogen molecule that result in abnormal fibrinogen function.

At least 25 single fibrinogen mutations (22 single nucleotide substitutions, one  
10 insertion and two deletions) are associated with increased thrombosis risk, and include the Thr312Ala polymorphism. At least thirteen mutations have been described in multiple reports from different unrelated kindreds and the remaining mutations have been unique to single families which makes them individual mutations.

Exemplary recurrent thrombophilic fibrinogen gene mutations and one common  
15 polymorphism related to venous thrombosis are shown in Table 1.

**Genetic predisposition:** Susceptibility of a subject to a genetic disease, such as venous thrombosis. However, such susceptibility may or may not result in actual development of the disease.

**Hybridization:** To form base pairs between complementary regions of two  
20 strands of DNA, RNA, or between DNA and RNA, thereby forming a duplex molecule. Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method and the composition and length of the hybridizing nucleic acid sequences. Generally, the temperature of hybridization and the ionic strength (such as the Na<sup>+</sup> concentration) of the hybridization buffer will  
25 determine the stringency of hybridization. Calculations regarding hybridization conditions for attaining particular degrees of stringency are discussed in Sambrook *et al.*, (1989) *Molecular Cloning*, second edition, Cold Spring Harbor Laboratory, Plainview, NY (chapters 9 and 11). The following is an exemplary set of hybridization conditions and is not limiting:

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Very High Stringency (detects sequences that share at least 90% identity)

Hybridization: 5x SSC at 65°C for 16 hours

Wash twice: 2x SSC at room temperature (RT) for 15 minutes each

Wash twice: 0.5x SSC at 65°C for 20 minutes each

5 High Stringency (detects sequences that share at least 80% identity)

Hybridization: 5x-6x SSC at 65°C-70°C for 16-20 hours

Wash twice: 2x SSC at RT for 5-20 minutes each

Wash twice: 1x SSC at 55°C-70°C for 30 minutes each

Low Stringency (detects sequences that share at least 50% identity)

10 Hybridization: 6x SSC at RT to 55°C for 16-20 hours

Wash at least twice: 2x-3x SSC at RT to 55°C for 20-30 minutes each.

**Insertion:** The addition of one or more nucleotides to a nucleic acid sequence, or the addition of one or more amino acids to a protein sequence.

15 **Isolated:** An “isolated” biological component (such as a nucleic acid molecule, protein, or organelle) has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, such as other chromosomal and extra-chromosomal DNA and RNA, proteins and organelles. Nucleic acid molecules and proteins that have been “isolated” include nucleic acid molecules and proteins purified by standard purification methods. The

20 term also embraces nucleic acid molecules and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acid molecules and proteins.

**Label:** An agent capable of detection, for example by ELISA, spectrophotometry, flow cytometry, or microscopy. For example, a label can be

25 attached to a nucleic acid molecule, thereby permitting detection of the nucleic acid molecule. Examples of labels include, but are not limited to, radioactive isotopes, enzyme substrates, co-factors, ligands, chemiluminescent agents, fluorophores, haptens, enzymes, and combinations thereof. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed for example in Sambrook *et al.*

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(Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1989) and Ausubel *et al.* (In Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1998).

**Methylenetetrahydrofolate reductase (MTHFR):** A protein that participates  
5 in the remethylation pathway of intracellular homocysteine metabolism. In the  
remethylation pathway catalyzed by methionine synthase, cobalamin acts as a cofactor  
and the methyl group is donated by 5-methyl-tetrahydrofolate, which derives from the  
reduction of 5, 10-methylenetetrahydrofolate by MTHFR. Includes the product of any  
MTHFR gene, cDNA, or RNA, or an MTHFR protein from any organism, such as a  
10 human. Examples include the mRNA sequence disclosed in GenBank Accession No.  
NM\_005957 (as well as the corresponding genomic and protein sequence).

The human MTHFR gene is located on chromosome 1p36.3, includes ~17 kb of  
DNA and has 11 exons. At least two polymorphisms in human MTHFR are associated  
with venous thrombosis: a 677 C→T polymorphism and a 1298 A→C polymorphism.

**Mutation:** Any change of a nucleic acid sequence as a source of genetic  
15 variation. For example, mutations can occur within a gene or chromosome, including  
specific changes in non-coding regions of a chromosome, for instance changes in or  
near regulatory regions of genes. Types of mutations include, but are not limited to,  
base substitution point mutations (such as transitions or transversions), deletions, and  
20 insertions. Missense mutations are those that introduce a different amino acid into the  
sequence of the encoded protein; nonsense mutations are those that introduce a new stop  
codon; and silent mutations are those that introduce the same amino acid often with a  
base change in the third position of codon. In the case of insertions or deletions,  
mutations can be in-frame (not changing the frame of the overall sequence) or frame  
25 shift mutations, which may result in the misreading of a large number of codons (and  
often leads to abnormal termination of the encoded product due to the presence of a stop  
codon in the alternative frame).

**Nucleic acid array:** An arrangement of nucleic acid molecules (such as DNA  
or RNA) in assigned locations on a matrix, such as that found in cDNA arrays, or

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oligonucleotide arrays.

**Nucleic acid molecules representing genes:** Any nucleic acid molecule, for example DNA (intron or exon or both), cDNA or RNA, of any length suitable for use as a probe or other indicator molecule, and that is informative about the corresponding  
5 gene.

**Nucleic acid molecules:** A deoxyribonucleotide or ribonucleotide polymer including, without limitation, cDNA, mRNA, genomic DNA, and synthetic (such as chemically synthesized) DNA. The nucleic acid molecule can be double-stranded or single-stranded. Where single-stranded, the nucleic acid molecule can be the sense  
10 strand or the antisense strand. In addition, nucleic acid molecule can be circular or linear.

The disclosure includes isolated nucleic acid molecules that include specified lengths of a VT-related nucleotide sequence. Such molecules can include at least 10, at least 15, at least 20, at least 21, at least 25, at least 30, at least 35, at least 40, at least 45 or  
15 at least 50 consecutive nucleotides of these sequences or more.

**Nucleotide:** Includes, but is not limited to, a monomer that includes a base linked to a sugar, such as a pyrimidine, purine or synthetic analogs thereof, or a base linked to an amino acid, as in a peptide nucleic acid (PNA). A nucleotide is one monomer in a polynucleotide. A nucleotide sequence refers to the sequence of bases in a  
20 polynucleotide.

**Oligonucleotide:** An oligonucleotide is a plurality of joined nucleotides joined by native phosphodiester bonds, between about 6 and about 300 nucleotides in length. An oligonucleotide analog refers to moieties that function similarly to oligonucleotides but have non-naturally occurring portions. For example, oligonucleotide analogs can contain  
25 non-naturally occurring portions, such as altered sugar moieties or inter-sugar linkages, such as a phosphorothioate oligodeoxynucleotide.

Particular oligonucleotides and oligonucleotide analogs can include linear sequences up to about 200 nucleotides in length, for example a sequence (such as DNA or RNA) that is at least 6 bases, for example at least 8, at least 10, at least 15, at least 20, at

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least 21, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 100 or even at least 200 bases long, or from about 6 to about 50 bases, for example about 10-25 bases, such as 12, 15, 20, 21, or 25 bases.

**Oligonucleotide probe:** A short sequence of nucleotides, such as at least 8, at  
5 least 10, at least 15, at least 20, at least 21, at least 25, or at least 30 nucleotides in length, used to detect the presence of a complementary sequence by molecular hybridization. In particular examples, oligonucleotide probes include a label that permits detection of oligonucleotide probe:target sequence hybridization complexes.

**Operably linked:** A first nucleic acid sequence is operably linked with a  
10 second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading  
15 frame.

**Open reading frame (ORF):** A series of nucleotide triplets (codons) coding for amino acids without any internal termination codons. These sequences are usually translatable into a peptide.

**Polymorphism:** As a result of mutations, a gene sequence may differ among  
20 individuals. The differing sequences are referred to as alleles. The alleles that are present at a given locus (a gene's location on a chromosome is termed as a locus) are referred to as the individual's genotype. Some loci vary considerably among individuals. If a locus has two or more alleles whose frequencies each exceed 1% in a population, the locus is said to be polymorphic. The polymorphic site is termed a  
25 polymorphism. The term polymorphism also encompasses variations that produce gene products with altered function, that is, variants in the gene sequence that lead to gene products that are not functionally equivalent. This term also encompasses variations that produce no gene product, an inactive gene product, or increased or decreased activity gene product or even no biological effect.

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Polymorphisms can be referred to, for instance, by the nucleotide position at which the variation exists, by the change in amino acid sequence caused by the nucleotide variation, or by a change in some other characteristic of the nucleic acid molecule or protein that is linked to the variation.

5           **Primers:** Short nucleic acid molecules, for instance DNA oligonucleotides 10 -  
100 nucleotides in length, such as about 15, 20, 21, 25, 30 or 50 nucleotides or more in  
length. Primers can be annealed to a complementary target DNA strand by nucleic acid  
hybridization to form a hybrid between the primer and the target DNA strand. Primer  
pairs can be used for amplification of a nucleic acid sequence, such as by PCR or other  
10   nucleic acid amplification methods known in the art.

          Methods for preparing and using nucleic acid primers are described, for example,  
in Sambrook *et al.* (In *Molecular Cloning: A Laboratory Manual*, CSHL, New York,  
1989), Ausubel *et al.* (ed.) (In *Current Protocols in Molecular Biology*, John Wiley &  
Sons, New York, 1998), and Innis *et al.* (*PCR Protocols, A Guide to Methods and*  
15   *Applications*, Academic Press, Inc., San Diego, CA, 1990). PCR primer pairs can be  
derived from a known sequence, for example, by using computer programs intended for  
that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical  
Research, Cambridge, MA). One of ordinary skill in the art will appreciate that the  
specificity of a particular primer increases with its length. Thus, for example, a primer  
20   including 30 consecutive nucleotides of a VT-related protein encoding nucleotide will  
anneal to a target sequence, such as another homolog of the designated VT-related  
protein, with a higher specificity than a corresponding primer of only 15 nucleotides.  
Thus, in order to obtain greater specificity, primers can be selected that includes at least  
20, at least 21, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50 or  
25   more consecutive nucleotides of a VT-related protein-encoding nucleotide sequences.

**Protein C (PC):** PC is activated after the binding of thrombin to its endothelial  
receptor, thrombomodulin. Activated PC inhibits clot formation by cleaving and  
inactivating factors Va and VIIIa. Includes the product of any PC gene, cDNA, or RNA,  
or a PC protein from any organism, such as a human. Examples include the mRNA

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sequence disclosed in GenBank Accession No. BC034377.1 (as well as the corresponding genomic and protein sequence).

The human PC gene is localized to human chromosome 2q13-14; it spans approximately 10 kb and contains nine exons. Loss-of-function mutations in the PC  
5 gene result in deficiency of PC, which is a well-established cause of VT. PC deficiency is classified into type I (low plasma concentrations of both functional and immunologic PC) and type II (low plasma levels of functional protein with normal antigen levels).

Among the at least 161 different PC gene mutations related to venous  
10 thrombosis in humans, at least 51 distinct mutations (48 point mutations, 2 deletions and 1 insertion) have been described in multiple unrelated kindreds and the remaining mutations are unique to single families which makes them individual mutations. At least forty recurrent mutations are associated with type I PC deficiency and 11 mutations have been found in patients with type II PC deficiency.

15 Three polymorphic sites (nt -1654C/T, -1641A/G and -1476A/T) located in the 5' untranslated region of the PC gene also have an effect on plasma PC levels. Subjects carrying the CGT allele have lower plasma PC levels than subjects with the other genotypes and this allele is a risk factor for venous thrombosis.

Exemplary recurrent PC gene mutations and polymorphisms related to  
20 venous thrombosis are shown in Table 1.

**Protein S (PS):** A non-enzymatic cofactor for activated PC in the proteolytic inactivation of factors Va and VIIIa. Includes the product of any PS gene, cDNA, or RNA, or a PS protein from any organism, such as a human. Examples include the mRNA sequence disclosed in GenBank Accession No. NM\_000313.1 (as well as the  
25 corresponding genomic and protein sequence).

Human DNA contains two PS genes: the active PROS1 gene and the pseudogene PRSO2, which map to 3p11.1-q11.2. PROS1 spans 80 kb genomic DNA and includes 15 exons and 14 introns. Loss-of-function mutations in PROS1 lead to a deficiency of PS. Three types of PS deficiency are recognized based on

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plasma measurements: type I is characterized by low total and free PS antigen levels, type II by decreased activity and normal total and free PS antigen levels and type III by a selective reduction in free PS levels.

Among at least 131 different PS gene mutations related to venous thrombosis in humans, at least 32 distinct mutations (25 point mutations, 3 deletions, 3 insertions, and 1 deletion and insertion) have been described in multiple unrelated kindreds and the remaining mutations have been unique to single families which makes them individual mutations. Twenty-five recurrent mutations have been reported to be associated with quantitative (type I and/or type III) PS deficiency, 3 mutations with qualitative (type II) PS deficiency and type of the PS deficiency could not be determined in the remaining 4 mutations, either because one of the plasma assays was missing or because the subject was on oral anticoagulant therapy. Four recurrent polymorphisms in the PS gene cosegregate with the deficient phenotype in families with hereditary PS deficiency.

Exemplary recurrent PS gene mutations and polymorphisms related to venous thrombosis are shown in Table 1.

**Prothrombin (Factor II, FII):** The precursor of serine protease thrombin, which is a vitamin K-dependent glycoprotein. Activated by FXa (in the presence of FVa and phospholipids), FIIa exhibits procoagulant, anticoagulant, and antifibrinolytic activities. Includes the product of any FII gene, cDNA, or RNA, or a FII protein from any organism, such as a human. Examples include the mRNA sequence disclosed in GenBank Accession No. V00595.1 (as well as the corresponding genomic and protein sequence).

The human gene coding for FII is localized on chromosome 11, band 11p11-q12 and spans 21 kb of DNA. The FII gene is organized in 14 exons, separated by 13 introns, with 5' and 3'-untranslated (UT) regions.

At least one single nucleotide substitution in the human FII gene is associated with increased thrombosis risk: G→ to A polymorphism at nucleotide 20210.

**Purified:** The term "purified" does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified protein preparation is one in

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which the protein referred to is more pure than the protein in its natural environment within a cell. For example, a preparation of a protein is purified such that the protein represents at least 50% of the total protein content of the preparation. Similarly, a purified oligonucleotide preparation is one in which the oligonucleotide is more pure than in an environment including a complex mixture of oligonucleotides.

**Recombinant:** A recombinant nucleic acid molecule is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination can be accomplished by chemical synthesis or by the artificial manipulation of isolated segments of nucleic acid molecules, such as by genetic engineering techniques.

**Sample:** A biological specimen, such as those containing genomic DNA, RNA (including mRNA), protein, or combinations thereof. Examples include, but are not limited to, peripheral blood, urine, saliva, tissue biopsy, surgical specimen, amniocentesis samples, and autopsy material.

**Sequence identity/similarity:** The identity/similarity between two or more nucleic acid sequences, or two or more amino acid sequences, is expressed in terms of the identity or similarity between the sequences. Sequence identity can be measured in terms of percentage identity; the higher the percentage, the more identical the sequences are. Sequence similarity can be measured in terms of percentage similarity (which takes into account conservative amino acid substitutions); the higher the percentage, the more similar the sequences are. Homologs or orthologs of nucleic acid or amino acid sequences possess a relatively high degree of sequence identity/similarity when aligned using standard methods. This homology is more significant when the orthologous proteins or cDNAs are derived from species which are more closely related (such as human and mouse sequences), compared to species more distantly related (such as human and *C. elegans* sequences).

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman & Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson &

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Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444, 1988; Higgins & Sharp, *Gene*, 73:237-44, 1988; Higgins & Sharp, *CABIOS* 5:151-3, 1989; Corpet *et al.*, *Nuc. Acids Res.* 16:10881-90, 1988; Huang *et al.* *Computer Appls. in the Biosciences* 8, 155-65, 1992; and Pearson *et al.*, *Meth. Mol. Bio.* 24:307-31, 1994. Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990) is available from several sources, including the National Center for Biological Information (NCBI, National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. Additional information can be found at the NCBI web site.

BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. To compare two nucleic acid sequences, the options can be set as follows: -i is set to a file containing the first nucleic acid sequence to be compared (such as C:\seq1.txt); -j is set to a file containing the second nucleic acid sequence to be compared (such as C:\seq2.txt); -p is set to blastn; -o is set to any desired file name (such as C:\output.txt); -q is set to -1; -r is set to 2; and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two sequences: C:\B12seq -i c:\seq1.txt -j c:\seq2.txt -p blastn -o c:\output.txt -q -1 -r 2.

To compare two amino acid sequences, the options of B12seq can be set as follows: -i is set to a file containing the first amino acid sequence to be compared (such as C:\seq1.txt); -j is set to a file containing the second amino acid sequence to be compared (such as C:\seq2.txt); -p is set to blastp; -o is set to any desired file name (such as C:\output.txt); and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two amino acid sequences: C:\B12seq -i c:\seq1.txt -j c:\seq2.txt -p blastp -o c:\output.txt. If the two compared sequences share homology, then the

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designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not share homology, then the designated output file will not present aligned sequences.

Once aligned, the number of matches is determined by counting the number of positions where an identical nucleotide or amino acid residue is presented in both sequences. The percent sequence identity is determined by dividing the number of matches either by the length of the sequence set forth in the identified sequence, or by an articulated length (such as 100 consecutive nucleotides or amino acid residues from a sequence set forth in an identified sequence), followed by multiplying the resulting value by 100. For example, a nucleic acid sequence that has 1166 matches when aligned with a test sequence having 1154 nucleotides is 75.0 percent identical to the test sequence (i.e.,  $1166 \div 1554 * 100 = 75.0$ ). The percent sequence identity value is rounded to the nearest tenth. For example, 75.11, 75.12, 75.13, and 75.14 are rounded down to 75.1, while 75.15, 75.16, 75.17, 75.18, and 75.19 are rounded up to 75.2. The length value will always be an integer. In another example, a target sequence containing a 20-nucleotide region that aligns with 20 consecutive nucleotides from an identified sequence as follows contains a region that shares 75 percent sequence identity to that identified sequence (that is,  $15 \div 20 * 100 = 75$ ).

```

                1                               20
20 Target Sequence:  AGGTCGTGTACTGTCAAGTCA
                   |  |  |  |  |  |  |  |  |
Identified Sequence: ACGTGGTGAAGTCCAGTGA

```

For comparisons of amino acid sequences of greater than about 30 amino acids, the Blast 2 sequences function is employed using the default BLOSUM62 matrix set to default parameters, (gap existence cost of 11, and a per residue gap cost of 1). Homologs are typically characterized by possession of at least 70% sequence identity counted over the full-length alignment with an amino acid sequence using the NCBI Basic Blast 2.0, gapped blastp with databases such as the nr or swissprot database. Queries searched with the blastn program are filtered with DUST (Hancock and Armstrong, 1994, *Comput. Appl.*

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*Biosci.* 10:67-70). Other programs use SEG. In addition, a manual alignment can be performed. Proteins with even greater similarity will show increasing percentage identities when assessed by this method, such as at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% sequence identity.

5           When aligning short peptides (fewer than around 30 amino acids), the alignment is be performed using the Blast 2 sequences function, employing the PAM30 matrix set to default parameters (open gap 9, extension gap 1 penalties). Proteins with even greater similarity to the reference sequence will show increasing percentage identities when assessed by this method, such as at least 60%, at least 70%, at least 75%, at least 80%, at  
10   least 85%, at least 90%, at least 95%, at least 98%, or at least 99% sequence identity. When less than the entire sequence is being compared for sequence identity, homologs will typically possess at least 75% sequence identity over short windows of 10-20 amino acids, and can possess sequence identities of at least 85%, at least 90%, at least 95% or at least 98% depending on their identity to the reference sequence. Methods for determining  
15   sequence identity over such short windows are described at the NCBI web site.

          One indication that two nucleic acid molecules are closely related is that the two molecules hybridize to each other under stringent conditions, as described above. Nucleic acid sequences that do not show a high degree of identity may nevertheless encode identical or similar (conserved) amino acid sequences, due to the degeneracy of the  
20   genetic code. Changes in a nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid molecules that all encode substantially the same protein. Such homologous nucleic acid sequences can, for example, possess at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, or at least 99% sequence identity determined by this method. An alternative (and not necessarily cumulative)  
25   indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

          One of skill in the art will appreciate that the particular sequence identity ranges are provided for guidance only; it is possible that strongly significant homologs could be

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obtained that fall outside the ranges provided.

**Single nucleotide polymorphism (SNP):** A single base (nucleotide) difference in a DNA sequence among individuals in a population. SNPs can be causative (actually involved in or influencing the condition or trait to which the SNP is linked) or associative (linked to but not having any direct involvement in or influence on the condition or trait to which the SNP is linked).

**Subject:** Living multi-cellular vertebrate organisms, a category that includes human and non-human mammals (such as veterinary subjects).

**Target sequence:** A sequence of nucleotides located in a particular region in a genome (such as a human genome or the genome of any mammal) that corresponds to one or more specific genetic abnormalities, such as one or more nucleotide substitutions, deletions, insertions, amplifications, or combinations thereof. The target can be for instance a coding sequence; it can also be the non-coding strand that corresponds to a coding sequence. Examples of target sequences include those sequences associated with venous thrombosis, such as those listed in Table 1.

**Venous thrombosis (VT):** A blood clot that forms within a vein. In particular examples, VT is associated with sluggish blood flow (for example as occurs during prolonged bed rest, pregnancy, and surgery) or with rapid coagulation of the blood. Examples include deep venous thromboses (DVTs) that form in the deep veins of the legs or in the pelvic veins. Such thrombi sometimes migrate to the lungs and form pulmonary emboli that lead to cardiopulmonary collapse and death.

**Venous thrombosis (VT)-related (or associated) molecule:** A molecule that is involved in the development of venous thrombosis. Such molecules include, for instance, nucleic acids (such as DNA, cDNA, or mRNAs) and proteins. Specific examples of VT-related molecules include those listed in Table 1, as well as fragments of the full-length genes or cDNAs that include the mutation(s), polymorphism(s), or both, responsible for increasing an individual's susceptibility to VT, and proteins and protein fragments encoded thereby.

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VT-related molecules can be involved in or influenced by venous thrombosis in many different ways, including causative (in that a change in a VT-related molecule leads to development of or progression to venous thrombosis) or resultive (in that development of or progression to venous thrombosis causes or results in a change in the  
5 VT-related molecule).

**Wild-type:** A genotype that predominates in a natural population of organisms, in contrast to that of mutant forms.

### **Mutations and Polymorphisms Involved in Venous Thrombosis**

10 Complex traits such as venous thrombosis can be understood by assuming an interaction between different mutations, polymorphisms, or both, in candidate susceptibility genes. The risk that is associated with each genetic defect may be relatively low in isolation but the simultaneous presence of several mutations or polymorphisms may dramatically increase disease susceptibility. Moreover,  
15 environmental factors can interact with one or more genetic variations to add further to the risk. Expression of a venous thrombosis phenotype is dependent on the interaction of gene products from several loci and environmental or acquired influences. Therefore, VT is a complex genetic disorder.

Several mutations and polymorphisms (such as one or more nucleotide  
20 substitutions, insertions, deletions, or combinations thereof) in genes associated with a risk of developing VT are known. However, a combination of mutations and polymorphisms (such as in genes statistically associated with VT) that permit accurate prediction of a subject's overall genetic predisposition to VT, in multiple ethnic groups, has not been previously identified.

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### ***Protein C, Protein S, and Antithrombin III***

Several genes involved in venous thrombosis, including protein C (PC), protein S (PS), and antithrombin III, are involved in anticoagulant pathways. PC and PS deficiencies result in defects in the activated PC anticoagulant system.

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At least 161 different detrimental PC gene mutations have been reported in humans (Reitsma *et al.*, *Thromb. Haemost.* 73:876-89, 1995). Among these 161 different PC gene mutations, only 51 distinct mutations (48 point mutations, 2 deletions and 1 insertion) have been described in multiple unrelated kindreds and the remaining  
5 109 mutations have been unique to single families which makes them individual mutations. Forty recurrent mutations are associated with type I PC deficiency and 11 mutations were observed in patients with type II PC deficiency. Three polymorphic sites (nt -1654C/T, -1641A/G and -1476A/T) are located in the 5'-untranslated region of the gene that have an effect on plasma PC levels.

10 PS deficiency has a highly heterogeneous molecular basis with at least 131 different mutations (Gandrille *et al.*, *Thromb. Haemost.* 84:918, 2000). Among all PS gene detrimental mutations, only 32 distinct mutations (25 point mutations, 3 deletions, 3 insertions, and 1 deletion and insertion) have been described in multiple unrelated kindreds and the remaining 100 mutations have been unique to single families which  
15 make them individual mutations. Twenty-five recurrent mutations have been reported to be associated with quantitative (type I and/or type III) PS deficiency, and 3 mutations with qualitative (type II) PS deficiency. Four recurrent polymorphisms in the PS gene cosegregate with the deficient phenotype in families with hereditary PS deficiency.

The prevalence of PC deficiency in the general population is approximately  
20 1/300. The carrier state for PC and PS deficiencies is associated with approximately a 10-fold increased thrombosis risk for VT. Homozygous PC and PS deficiency is usually associated with a severe clinical phenotype known as purpura fulminans, characterized by extensive thromboses in the microcirculation early after birth.

Heterozygous antithrombin III (AT III) deficiency is associated with increased  
25 risk for VT. There are at least 127 distinct defects (Lane *et al.*, *Thromb. Haemost.* 77:197-211, 1997) associated with AT III deficiency: 92 mutations for type I AT III deficiency (40 point mutations, 40 small insertions or deletions and 12 large deletions) and 35 mutations for type II AT III deficiency (12 RS, 12 HBS and 11 PE mutations, all point mutations). Among the type I mutations, only 11 distinct mutations (7 point

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mutations and 4 deletions or insertions) have been described in multiple unrelated kindreds and the remaining 81 mutations have been unique to single families which makes them individual mutations. In type II, 19 of the 35 mutations (seven RS, six HBS and six PE mutations) have been described in multiple unrelated kindreds and the remaining 16 have been reported to be individual mutations.

The prevalence of AT III deficiency in the general population ranges from 0.2/1000 to 18/1000. In a population-based control study, a five-fold increased risk for VT linked AT III deficiency was reported. The prevalence of AT III deficiency in thrombosis patients ranges from 1% to 8%.

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#### ***Factor V, prothrombin, and fibrinogen***

Other genes involved in venous thrombosis include Factor V (FV), prothrombin (Factor II), and fibrinogen, which are involved in procoagulant pathways. Altered activity of mutated FV is the most common hereditary blood coagulation disorder that affects development of VT (Nicolaes *et al.*, *Arterioscler. Thromb. Vasc. Biol.* 22:530-8, 2002). Downregulation of the procoagulant activity of activated FV (FVa) is accomplished by activated protein C (APC)-mediated proteolysis of FVa at three different sequential cleavage sites: Arg 506, Arg 306, and Arg 679 (the numbering of nucleotides or amino acids herein refer to human genes). A defect at one or more of these three cleavage sites can affect the APC inactivation process even though procoagulation activity may remain normal.

At least five recurrent single nucleotide substitutions in the human FV gene are associated with increased thrombosis risk. In 90% of cases, resistance to APC due to a single nucleotide substitution (FV Leiden; 1691G→A) that results in the replacement of Arg506 with Gln (R506Q). Its prevalence in Caucasian populations is approximately 5% and is as high as 20% to 40% in patients with VT. However, very few cases of FV Leiden have been reported among other races and it has not been found in South East Asia and Africa, so it is believed that FV Leiden mutation is specific to Caucasians (Takamiya *et al.*, *Thromb. Haemost.* 74:996, 1995; Fujimura *et al.*, *Thromb.*

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*Haemost.* 74:1381-2, 1995; Chan *et al.*, *Thromb. Haemost.* 75:522-3, 1996).

Another single nucleotide substitution in the FV gene, R485K, is associated with increased thrombosis risk in Far East populations (Hiyoshi *et al.*, *Thromb. Haemost.* 80:705-6, 1998; Le *et al.*, *Clin. Genet.* 57:296-303, 2000). The R485K polymorphism is  
5 a G → A transition occurring at nucleotide 1628 and results in the replacement of the codon AGA of Arg 485 by an AAA codon predicting a Lys residue. Although the frequency of the K485 allele is low in Caucasians and high in Asians and high in Asians and Africans, this polymorphism was shown to be associated with increased thrombosis risk in both Far East and Caucasian populations (Faisel *et al.*, *Eur. J. Hum. Genet.*  
10 12:187-91, 2004)

Three other single nucleotide substitutions are associated with increased risk of thrombosis in different populations. Two mutations in exon 7 of the human FV gene affect the Arg306 APC cleavage site. These two mutations also have a heterogeneous racial distribution. The FV Cambridge mutation is a G to C transition at nucleotide  
15 position 1091 and predicts replacement of arginine with a threonine at amino acid position 306 (Arg306Thr). This mutation has only been described in Caucasian populations (Franco *et al.*, *Thromb. Haemost.* 81:312-3, 1999). The second mutation, FV Hong Kong, is an A to G transition at nucleotide position 1090 and changes Arg306 to Gly. Although this mutation was originally described in Chinese populations, it has a  
20 prevalence of 0.4% in Caucasians (Franco *et al.*, *Thromb. Haemost.* 81:312-3, 1999).

Another single nucleotide substitution in exon 13 of the human FV gene, referred to as the R2 allele, is an A to G transition at nucleotide position 4070, which replaces His by Arg at position 1299 (H1299R). The prevalence of R2 allele is significantly higher in the patients with VT than in the healthy controls, with respective  
25 values of 18.5% and 11.4% (Alhenc-Gelas *et al.*, *Thromb. Haemost.* 81:193-7, 1999). This polymorphism has a prevalence of 11.9% in U.S. Caucasians, 5.6% in African-Americans, 13.4% in Asian or Pacific Islanders and 11.3% in Hispanics (Benson *et al.*, *Thromb. Haemost.* 86:1188-92, 2001).

At least one single nucleotide substitution is associated with increased

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thrombosis risk in the prothrombin (Factor II, FII) gene. The G→ to A polymorphism at nucleotide 20210 in the 3'-UT region of the prothrombin gene is the second most frequently inherited risk factor for venous thrombosis (Poort *et al.*, *Blood* 88:3698-703, 1996). FII G20210A is associated with hyperprothrombinemia and a two- to five fold  
5 increased risk of VT. It is found in 1%-3% of subjects in healthy subjects and in 6%-18% of patients with VT in Caucasian populations (Rosendaal *et al.*, *Thromb. Haemost.* 79:706-8, 1998), but is quite rare in African Americans and Amerindians from Brazil (Dilley *et al.*, *Blood* 90:652a, 1997; Arruda *et al.*, *Thromb. Haemost.* 78:1430-3, 1997). This mutation has not been found in West African, Amazonian Indian, Australasian,  
10 Latin American, Japanese or Chinese subjects (Ferraresi *et al.*, *Arterioscler. Thromb. Vasc. Biol.* 17:2418-22, 1997; Rahimy *et al.*, *Thromb. Haemost.* 79:444-5, 1998; Isshiki *et al.*, *Blood Coagul. Fibrinol.* 9:105-6, 1998; Miyata *et al.*, *Blood Coagul. Fibrinol.* 9:451-2, 1998, Rees *et al.* *Br. J. Haematol.* 105:564-566, 1999).

At least 25 thrombophilic fibrinogen mutations (22 single nucleotide  
15 substitutions, 1 insertion and two deletions) are associated with VT (De Stefano *et al.*, *Br. J. Haematol.* 106:564-8, 1999). At least thirteen of the mutations are from different unrelated kindreds; the remaining mutations are unique to single families, making them individual mutations. The prevalence of inherited dysfibrinogenemia among the general population is unknown; however, the prevalence among patients with a history of  
20 venous thrombosis is 0.8 % (Carter *et al.*, *Blood* 96:1177-9, 2000). A common polymorphism leading to a substitution of threonine by alanine at codon 312 (Thr312Ala polymorphism) within the carboxy-terminal end of the fibrinogen A $\alpha$  chain is associated with venous thromboembolism via influencing clot stability and predisposing clots to embolization in the venous vascular trees (Carter *et al.*, *Blood*  
25 96:1177-9, 2000; Standeven *et al.*, *Circulation* 107:2326-30, 2003; Hayes, *Arch. Pathol. Lab. Med.* 126:1387-90, 2002). The polymorphism is observed in 51% of patients with pulmonary embolism and in 40% of healthy subjects. No differences have been found in genotype distribution for Thr312Ala polymorphism in Caucasians and Asians and this polymorphism is associated with elevated fibrinogen levels in both populations (Liu

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*et al.*, *J. Med. Genet.* 38:31-5, 2001; Kain *et al.*, *Am. J. Epidemiol.* 156:174-9, 2002).

***Angiotensin I-converting enzyme and methylenetetrahydrofolate reductase***

Additional genes involved in venous thrombosis include, but are not limited to,  
5 angiotensin I-converting enzyme (ACE) and methylenetetrahydrofolate reductase  
(MTHFR).

The renin angiotensin system affects hemostasis through different mechanisms.  
In intron 16 of the human ACE gene, a polymorphism consisting of an insertion or  
deletion of a 288-bp fragment is known (Rigat *et al.*, *Nuc. Acids Res.* 20:1433, 1992).  
10 The ACE DD genotype is associated with increased levels of circulating enzyme and 3  
to 10-fold increased risk to venous thromboembolism among Caucasians and African-  
Americans. The ACE DD genotype has also been reported in the Japanese population.

Mild-to-moderate hyperhomocysteinemia (fasting levels of total homocysteine  
between 15 and 100  $\mu\text{mol/l}$ ) is an established risk factor for VT and is associated with  
15 two- to four fold increased risk of thrombosis. Although it can be caused by several  
acquired causes including nutritional deficiencies of vitamin B12, vitamin B6 and  
folate, advanced age, chronic renal failure and the use of anti-folic drugs, two common  
polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene are  
associated with mild-to moderate hyperhomocysteinemia (Cattaneo, *Thromb. Haemost.*  
20 81:165-76, 1999; Franco and Reitsma, *Hum. Genet.* 109:369-84, 2001).

MTHFR 677 C $\rightarrow$ T polymorphism is located in human exon 4 at the folate  
binding site, converting an alanine into a valine. In its homozygous state, C677T  
polymorphism is associated with thermolability of MTHFR, leading to 60-70%  
reduction of the enzymatic activity and mild to moderate hyperhomocysteinemia  
25 (Franco and Reitsma, *Hum. Genet.* 109:369-84, 2001; Frosst *et al.*, *Nat. Genet.* 10:111-  
3, 1995). The C677T polymorphism in human MTHFR has a relatively high frequency  
throughout the world, TT genotype is present in about 5% to 17% of the general  
population with a very heterogeneous distribution among different ethnic groups,  
highest prevalence in Europe and lowest prevalence in Africa (Frosst *et al.*, *Nat. Genet.*

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10:111-3, 1995; Schneider *et al.*, *Am. J. Hum. Genet.* 62:1258-60, 1998; De Franchis *et al.*, *Am. J. Hum. Genet.* 59:262-4, 1996; Ma *et al.*, *Circulation* 94:2410-6, 1996; Deloughery *et al.*, *Circulation* 94:3074-8, 1996; Arruda V *et al.*, *Thromb. Haemost.* 77:818-21, 1997). Homozygous MTHFR C677T polymorphism is an independent risk  
5 factor for venous thrombosis with a prevalence of 11%-27% in venous thrombosis patients in Caucasians but not associated with VT in Asians and Africans (Arruda *et al.*, *Thromb. Haemost.* 77:818-21, 1997; Margaglione *et al.*, *Thromb. Haemost.* 79:907-11, 1998; Salomon *et al.*, *Arterioscler. Thromb. Vasc. Biol.* 19:511-8, 1999).

Another MTHFR polymorphism, 1298 A→C, is in human exon 7 within the  
10 presumptive regulatory domain and converts a glutamine into an alanine. By itself, this polymorphism does not appear to be associated with hyperhomocysteinemia but compound heterozygosity with MTHFR 677 C→T results in decreased enzyme activity and increased homocysteine levels (Weisberg *et al.*, *Mol. Genet. Metab.* 68:511-2, 1999).

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### **Determining Genetic Predisposition to Venous Thrombosis**

Provided herein are methods of determining whether a subject, such as an otherwise healthy subject, or a subject suspected or at risk of developing thrombi, is susceptible to developing venous thrombosis (VT). The methods involve detecting an  
20 abnormality (such as a mutation or polymorphism) in at least one VT-related molecule of the subject, such as a nucleic acid molecule that encodes a coagulation-related protein. Specific encompassed embodiments include diagnostic or prognostic methods in which one or more mutations or polymorphisms in a VT-related nucleic acid molecule in cells of the individual is detected. In particular embodiments, an  
25 abnormality is detected in a subset of VT-related molecules (such as nucleic acid sequences), or all known VT-related molecules, that selectively detect a genetic predisposition of a subject to develop VT.

In particular examples, the subset of molecules includes a set of 10 VT-related susceptibility alleles associated with venous thrombotic events, wherein the 10 VT-

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related susceptibility alleles are present in at least 95% of Caucasians subjects who are at risk for (or who have experienced) a venous thrombosis. In particular examples, the 10 VT-related susceptibility alleles are present in at least 98% of Caucasians, such as at least 99%, and at least 82% of Asians and African populations, such as at least 85% of  
5 Africans who have or are at risk of developing a VT.

In yet other examples, the number of VT-related susceptibility alleles screened is at least 10, for example at least 15, at least 20, at least 50, at least 100, at least 143, at least 200, at least 287, or even at least 500 alleles. In other examples, the methods employ screening no more than 600, no more than 500, no more than 400, no more than  
10 287, no more than 200, no more than 143, no more than 100, no more than 50, or no more than 10 VT-related susceptibility alleles. Examples of particular VT-related susceptibility alleles are shown in Table 1.

As used herein, the term "VT-related molecule" includes VT-related nucleic acid molecules (such as DNA, RNA or cDNA) and VT-related proteins. The term is not  
15 limited to those molecules listed in Table 1 (and molecules that correspond to those listed), but also includes other nucleic acid molecules and proteins that are influenced (such as to level, activity, localization) by or during venous thrombosis, including all of such molecules listed herein.

Examples of VT-related genes include antithrombin III, protein C, protein S,  
20 fibrinogen, factor V, prothrombin (factor II), methylenetetrahydroflolate reductase (MTHFR) and angiotensin-I converting enzyme (ACE). In certain examples, abnormalities are detected in at least one VT-related nucleic acid, for instance in at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 10, at least 15 or more VT-related nucleic acid molecules. In particular examples, certain of the  
25 described methods employ screening no more than 100, no more than 50, no more than 40, no more than 30, no more than 20, or no more than 15 VT-related genes.

This disclosed method (MERT) provides a rapid, straightforward, accurate and affordable multiple genetic screening method for screening in one assay overall inherited venous thrombosis susceptibility, that has a high predictive power for

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identification of asymptomatic carriers. It allows early recognition of subjects who may require prophylactic anticoagulant therapy during high risk situations, such as pregnancy, puerperium, use of oral contraceptives or hormone replacement therapy, trauma, surgery, fractures, prolonged immobilization, long air journeys (such as those  
5 more than 4 hours), advanced age, antiphospholipid antibodies, previous thrombosis history, myeloproliferative disorders, malignancy, or combinations thereof. The disclosed assay can be used to reduce the yearly incidence of venous thrombosis by early identification of individuals at inherited risk. By detecting individuals before they develop symptoms, effective preventive measures, such as early thromboprophylaxis or  
10 even decisions such as avoiding the use of oral contraceptives or hormone replacement therapy, can be instituted.

As discussed above, there are differences in the causes of the inherited venous thrombosis among different ethnic groups. Whereas FV Leiden and prothrombin G0210A polymorphisms are the most prevalent risk factors for venous thrombosis in  
15 Caucasians, Asian and African patients exhibit no or very rare FV Leiden or prothrombin G20210A polymorphisms. The disclosed methods and arrays are designed to determine inherited venous thrombophilia risk not only in Caucasians but also diverse ethnic populations. In one particular example, the method has a high predictive power in different ethnic populations (such as at least 98% for Caucasians, at least 84% for  
20 Asians and at least 87% for Africans). In other examples, the method detects abnormalities in VT-related molecules (such as nucleic acid sequences) wherein the abnormalities are found in at least 99% of Caucasians, at least 85% of Asians, and at least 88% of Africans who have had a VT. Therefore, the applicability of the disclosed methods and arrays in diverse ethnic populations makes it a powerful approach.

25 In particular examples, the disclosed methods and arrays are cost-effective compared to the currently available plasma-based thrombophilia screening panel, which includes antigenic and activity based determination of protein C and S, AT III antigen and activity, thrombin time and reptilase time for dysfibrinogenemia, quantitative determination of fibrinogen level and PCR-based direct mutation analysis of FV Leiden,

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prothrombin 20210A and MTHFR polymorphisms.

For example, the disclosed method provides advantages over AT III deficiency assays, because the disclosed method detects type I and II deficiencies in one assay instead of two subsequent tests, and detects variant AT III type II defects that can  
5 unfortunately be missed due to long incubation times of many automated functional heparin cofactor assay analyzers used in clinical practice, and by avoiding high rates of false positivity.

In other examples, the disclosed method provides advantages over PC deficiency assays, including the clotting assay of functional PC level, immunological PC assay and  
10 chromogenic assay of PC activity, because the disclosed method can in one embodiment detect type I and II deficiencies in one assay instead of three subsequent tests and overcomes the difficulty of distinguishing healthy subjects from asymptomatic PC deficient individuals due to the presence of a significant overlap between low normal levels and mild PC deficiency, by avoiding the under-determination of PC deficient  
15 individuals because of the increase in PC concentration as a function of age which is approximately 4% per decade, and by avoiding high rates of false positivity.

In other examples, the disclosed method provides advantages over PS deficiency assays, which include clotting assay of functional PS level, immunoassay of PS, and enzyme-linked immunosorbent assays for total and free PS measurements because the  
20 disclosed method can in one embodiment detect quantitative and functional defects in one assay instead of four subsequent phenotypic assays and overcomes the difficulty in the diagnosis of PS deficiency with the immunologic assay which is complicated by the presence of two molecular forms of PS in the plasma (free PS and C4b-BP/PS complexes), overcomes the difficulty of distinguishing healthy subjects from  
25 asymptomatic PS deficient individuals due to the presence of overlapping values between controls and PS deficient individuals, especially those with type III PS deficiency, and by avoiding high rates of false positivity.

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In other examples, the disclosed method provides an advantage over assays for dysfibrinogenemia which include thrombin time and reptilase time as first line tests, because the disclosed method avoids high rates of false positivity.

## 5 *Clinical Specimens*

Appropriate specimens for use with the current disclosure in determining a subject's genetic predisposition to VT include any conventional clinical samples, for instance blood or blood-fractions (such as serum). Techniques for acquisition of such samples are well known in the art (for example see Schluger *et al. J. Exp. Med.*  
10 176:1327-33, 1992, for the collection of serum samples). Serum or other blood fractions can be prepared in the conventional manner. For example, about 200  $\mu$ L of serum can be used for the extraction of DNA for use in amplification reactions.

Once a sample has been obtained, the sample can be used directly, concentrated (for example by centrifugation or filtration), purified, or combinations thereof, and an  
15 amplification reaction performed. For example, rapid DNA preparation can be performed using a commercially available kit (such as the InstaGene Matrix, BioRad, Hercules, CA; the NucliSens isolation kit, Organon Teknika, Netherlands). In one example, the DNA preparation method yields a nucleotide preparation that is accessible to, and amenable to, nucleic acid amplification.

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### *Amplification of nucleic acid molecules*

The nucleic acid samples obtained from the subject to obtain amplification products, including sequences from AT III, protein C, protein S, fibrinogen, factor V, prothrombin (factor II), MTHFR, and ACE can be amplified from the clinical sample  
25 prior to detection. In one example, DNA sequences are amplified. In another example, RNA sequences are amplified.

Any nucleic acid amplification method can be used. In one specific, non-limiting example, polymerase chain reaction (PCR) is used to amplify the nucleic acid sequences associated with venous thrombosis. Other exemplary methods include, but

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are not limited to, RT-PCR and transcription-mediated amplification (TMA).

The target sequences to be amplified from the subject include AT III, protein C, protein S, fibrinogen, factor V, factor II, ACE, and MTHFR. In particular examples, the VT-associated target sequences to be amplified consist essentially of, or consist only of  
5 AT III, protein C, protein S, fibrinogen, factor V, factor II, MTHFR and ACE.

A pair of primers can be utilized in the amplification reaction. One or both of the primers can be labeled, for example with a detectable radiolabel, fluorophore, or biotin molecule. The pair of primers includes an upstream primer (which binds 5' to the downstream primer) and a downstream primer (which binds 3' to the upstream primer).  
10 The pair of primers used in the amplification reaction are selective primers which permit amplification of a nucleic acid involved in venous thrombosis. Primers can be selected to amplify a nucleic acid molecule listed in Table 1, or represented by those listed in Table 1.

An additional pair of primers can be included in the amplification reaction as an  
15 internal control. For example, these primers can be used to amplify a "housekeeping" nucleic acid molecule, and serve to provide confirmation of appropriate amplification. In another example, a target nucleic acid molecule including primer hybridization sites can be constructed and included in the amplification reactor. One of skill in the art will readily be able to identify primer pairs to serve as internal control primers.

20

#### ***Arrays for detecting nucleic acid and protein sequences***

In particular examples, methods for detecting an abnormality in at least one VT-related gene use the arrays disclosed herein. Such arrays can include nucleic acid molecules. In one example, the array includes nucleic acid oligonucleotide probes that  
25 can hybridize to wild-type, mutant, or polymorphic VT gene sequences, such as AT III, protein C, protein S, fibrinogen, factor V, prothrombin (factor II), MTHFR and ACE. In a particular example, an array includes oligonucleotides that can recognize the 143 VT-associated recurrent mutations and polymorphisms listed in Table 1, such as the oligonucleotide probes shown in even numbered SEQ ID NOS: 2-284 and SEQ ID NO:

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287. In other examples, an array includes oligonucleotide probes that can recognize both mutant and wild-type factor V, prothrombin (factor II), AT III, PC, PS, fibrinogen, MTHFR, and ACE sequences, such as SEQ ID NOS: 1-287. Certain of such arrays (as well as the methods described herein) can include VT-related molecules that are not listed in Table 1, as well as other sequences, such as one or more probes that recognize one or more housekeeping genes.

Arrays can be used to detect the presence of amplified sequences involved in venous thrombosis, such as antithrombin III, protein C, protein S, fibrinogen, factor V, prothrombin (factor II), MTHFR and ACE sequences, using specific oligonucleotide probes. The arrays herein termed "VT detection arrays," are used to determine the genetic susceptibility of a subject to developing venous thrombosis. In one example, a set of oligonucleotide probes is attached to the surface of a solid support for use in detection of the VT-associated sequences, such as those amplified nucleic acid sequences obtained from the subject. Additionally, if an internal control nucleic acid sequence was amplified in the amplification reaction (see above), an oligonucleotide probe can be included to detect the presence of this amplified nucleic acid molecule.

The oligonucleotide probes bound to the array can specifically bind sequences amplified in the amplification reaction (such as under high stringency conditions). Thus, sequences of use with the method are oligonucleotide probes that recognize the VT-related sequences, such as antithrombin III, PC, PS, fibrinogen, factor V, prothrombin (factor II), MTHFR and ACE gene sequences. Such sequences can be determined by examining the sequences of the different species, and choosing primers that specifically anneal to a particular wild-type or mutant sequence (such as those listed in Table 1 or represented by those listed in Table 1), but not others. One of skill in the art will be able to identify other VT-associated oligonucleotide molecules that can be attached to the surface of a solid support for the detection of other amplified VT-associated nucleic acid sequences.

The methods and apparatus in accordance with the present disclosure takes advantage of the fact that under appropriate conditions oligonucleotides form base-

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paired duplexes with nucleic acid molecules that have a complementary base sequence. The stability of the duplex is dependent on a number of factors, including the length of the oligonucleotides, the base composition, and the composition of the solution in which hybridization is effected. The effects of base composition on duplex stability may be  
5 reduced by carrying out the hybridization in particular solutions, for example in the presence of high concentrations of tertiary or quaternary amines.

The thermal stability of the duplex is also dependent on the degree of sequence similarity between the sequences. By carrying out the hybridization at temperatures close to the anticipated  $T_m$ 's of the type of duplexes expected to be formed between the  
10 target sequences and the oligonucleotides bound to the array, the rate of formation of mis-matched duplexes may be substantially reduced.

The length of each oligonucleotide sequence employed in the array can be selected to optimize binding of target VT-associated nucleic acid sequences. An optimum length for use with a particular VT-associated nucleic acid sequence under  
15 specific screening conditions can be determined empirically. Thus, the length for each individual element of the set of oligonucleotide sequences including in the array can be optimized for screening. In one example, oligonucleotide probes are from about 20 to about 35 nucleotides in length or about 25 to about 40 nucleotides in length.

The oligonucleotide probe sequences forming the array can be directly linked to  
20 the support, for example via the 5'- or 3'-end of the probe. In one example, the oligonucleotides are bound to the solid support by the 5' end. However, one of skill in the art can determine whether the use of the 3' end or the 5' end of the oligonucleotide is suitable for bonding to the solid support. In general, the internal complementarity of an oligonucleotide probe in the region of the 3' end and the 5' end determines binding to  
25 the support. Alternatively, the oligonucleotide probes can be attached to the support by non-VT-associated sequences such as oligonucleotides or other molecules that serve as spacers or linkers to the solid support.

In another example, an array includes protein sequences, which include at least one VT-related protein such as one encoded by a nucleic acid molecule listed in Table 1

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(or genes, cDNAs or other polynucleotide molecules including one of the listed sequences, or a fragment thereof), or a fragment of such protein, or an antibody specific to such a protein or protein fragment. Such arrays can also contain any particular subset of the nucleic acids (or corresponding molecules) listed in Table 1. The proteins or  
5 antibodies forming the array can be directly linked to the support. Alternatively, the proteins or antibodies can be attached to the support by spacers or linkers to the solid support.

Abnormalities in VT-related proteins can be detected using, for instance, a VT protein-specific binding agent, which in some instances will be detectably labeled. In  
10 certain examples, therefore, detecting an abnormality includes contacting a sample from the subject with a VT protein-specific binding agent; and detecting whether the binding agent is bound by the sample and thereby measuring the levels of the VT-related protein present in the sample, in which a difference in the level of VT-related protein in the  
15 sample, relative to the level of VT-related protein found in an analogous sample from a subject not predisposed to developing VT, or a standard VT-related protein level in analogous samples from a subject not having a predisposition for developing VT, is an abnormality in that VT-related molecule.

In particular examples, the microarray material is formed from glass (silicon dioxide). Suitable silicon dioxide types for the solid support include, but are not limited  
20 to: aluminosilicate, borosilicate, silica, soda lime, zinc titania and fused silica (for example see Schena, *Microarray Analysis*. John Wiley & Sons, Inc, Hoboken, New Jersey, 2003). The attachment of nucleic acids to the surface of the glass can be achieved by methods known in the art, for example by surface treatments that form from an organic polymer. Particular examples include, but are not limited to: polypropylene,  
25 polyethylene, polybutylene, polyisobutylene, polybutadiene, polyisoprene, polyvinylpyrrolidone, polytetrafluoroethylene, polyvinylidene difluoride, polyfluoroethylene-propylene, polyethylenevinyl alcohol, polymethylpentene, polychlorotrifluoroethylene, polysulfones, hydroxylated biaxially oriented polypropylene, aminated biaxially oriented polypropylene, thiolated biaxially oriented

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polypropylene, ethyleneacrylic acid, thylene methacrylic acid, and blends of copolymers thereof (see U.S. Patent No. 5,985,567, herein incorporated by reference), organosilane compounds that provide chemically active amine or aldehyde groups, epoxy or polylysine treatment of the microarray. Another example of a solid support surface is  
5 polypropylene.

In general, suitable characteristics of the material that can be used to form the solid support surface include: being amenable to surface activation such that upon activation, the surface of the support is capable of covalently attaching a biomolecule such as an oligonucleotide thereto; amenability to "in situ" synthesis of biomolecules;  
10 being chemically inert such that at the areas on the support not occupied by the oligonucleotides are not amenable to non-specific binding, or when non-specific binding occurs, such materials can be readily removed from the surface without removing the oligonucleotides.

In one example, the surface treatment is amine-containing silane derivatives.  
15 Attachment of nucleic acids to an amine surface occurs via interactions between negatively charged phosphate groups on the DNA backbone and positively charged amino groups (Schna, Microarray Analysis. John Wiley & Sons, Inc, Hoboken, New Jersey, 2003, herein incorporated by reference). In another example, reactive aldehyde groups are used as surface treatment. Attachment to the aldehyde surface is achieved by  
20 the addition of 5'-amine group or amino linker to the DNA of interest. Binding occurs when the nonbonding electron pair on the amine linker acts as a nucleophile that attacks the electropositive carbon atom of the aldehyde group (Id.).

A wide variety of array formats can be employed in accordance with the present disclosure. One example includes a linear array of oligonucleotide bands, generally  
25 referred to in the art as a dipstick. Another suitable format includes a two-dimensional pattern of discrete cells (such as 4096 squares in a 64 by 64 array). As is appreciated by those skilled in the art, other array formats including, but not limited to slot (rectangular) and circular arrays are equally suitable for use (see U.S. Patent No. 5,981,185, herein incorporated by reference). In one example, the array is formed on a

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polymer medium, which is a thread, membrane or film. An example of an organic polymer medium is a polypropylene sheet having a thickness on the order of about 1 mil. (0.001 inch) to about 20 mil., although the thickness of the film is not critical and can be varied over a fairly broad range. Particularly disclosed for preparation of arrays  
5 at this time are biaxially oriented polypropylene (BOPP) films; in addition to their durability, BOPP films exhibit a low background fluorescence. In a particular example, the array is a solid phase, Allele-Specific Oligonucleotides (ASO) based nucleic acid array.

The array formats of the present disclosure can be included in a variety of  
10 different types of formats. A "format" includes any format to which the solid support can be affixed, such as microtiter plates, test tubes, inorganic sheets, dipsticks, and the like. For example, when the solid support is a polypropylene thread, one or more polypropylene threads can be affixed to a plastic dipstick-type device; polypropylene membranes can be affixed to glass slides. The particular format is, in and of itself,  
15 unimportant. All that is necessary is that the solid support can be affixed thereto without affecting the functional behavior of the solid support or any biopolymer absorbed thereon, and that the format (such as the dipstick or slide) is stable to any materials into which the device is introduced (such as clinical samples and hybridization solutions).

20 The arrays of the present disclosure can be prepared by a variety of approaches. In one example, oligonucleotide or protein sequences are synthesized separately and then attached to a solid support (see U.S. Patent No. 6,013,789, herein incorporated by reference). In another example, sequences are synthesized directly onto the support to provide the desired array (see U.S. Patent No. 5,554,501, herein incorporated by  
25 reference). Suitable methods for covalently coupling oligonucleotides and proteins to a solid support and for directly synthesizing the oligonucleotides or proteins onto the support are known to those working in the field; a summary of suitable methods can be found in Matson *et al.*, *Anal. Biochem.* 217:306-10, 1994. In one example, the oligonucleotides are synthesized onto the support using conventional chemical

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techniques for preparing oligonucleotides on solid supports (such as see PCT applications WO 85/01051 and WO 89/10977, or U.S. Patent No. 5,554,501, herein incorporated by reference).

A suitable array can be produced using automated means to synthesize  
5 oligonucleotides in the cells of the array by laying down the precursors for the four bases in a predetermined pattern. Briefly, a multiple-channel automated chemical delivery system is employed to create oligonucleotide probe populations in parallel rows (corresponding in number to the number of channels in the delivery system) across the substrate. Following completion of oligonucleotide synthesis in a first direction, the  
10 substrate can then be rotated by 90° to permit synthesis to proceed within a second (2°) set of rows that are now perpendicular to the first set. This process creates a multiple-channel array whose intersection generates a plurality of discrete cells.

In particular examples, the oligonucleotide probes on the array include one or more labels, that permit detection of oligonucleotide probe:target sequence  
15 hybridization complexes.

### ***Detection of nucleic acids and proteins***

The nucleic acids and proteins obtained from the subject may contain one or more insertions, deletions, substitutions, or combinations thereof in one or more genes  
20 associated with venous thrombosis, such as those listed in Table 1. Such mutations or polymorphisms (or both) can be detected to determine if the subject has a genetic disposition to developing venous thrombosis. Any method of detecting a nucleic acid molecule or protein can be used, such as physical or functional assays.

Methods for labeling nucleic acid molecules and proteins, such that they can be  
25 detected, are well known. Examples of such labels include non-radiolabels and radiolabels. Non-radiolabels include, but are not limited to an enzyme, chemiluminescent compound, fluorescent compound (such as FITC, Cy3, and Cy5), metal complex, hapten, enzyme, colorimetric agent, a dye, or combinations thereof. Radiolabels include, but are not limited to, <sup>125</sup>I and <sup>35</sup>S. For example, radioactive and

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fluorescent labeling methods, as well as other methods known in the art, are suitable for use with the present disclosure. In one example, the primers used to amplify the subject's nucleic acids are labeled (such as with biotin, a radiolabel, or a fluorophore). In another example, the amplified nucleic acid samples are end-labeled to form labeled amplified material. For example, amplified nucleic acid molecules can be labeled by including labeled nucleotides in the amplification reactions. In a particular example, proteins obtained from a subject are labeled and subsequently analyzed, for example by applying them to an array.

The amplified nucleic acid molecules associated with venous thrombosis are applied to the VT detection array under suitable hybridization conditions to form a hybridization complex. In particular examples, the amplified nucleic acid molecules include a label. In one example, a pre-treatment solution of organic compounds, solutions that include organic compounds, or hot water, can be applied before hybridization (see U.S. Patent No. 5,985,567, herein incorporated by reference).

Hybridization conditions for a given combination of array and target material can be optimized routinely in an empirical manner close to the  $T_m$  of the expected duplexes, thereby maximizing the discriminating power of the method. Identification of the location in the array, such as a cell, in which binding occurs, permits a rapid and accurate identification of sequences associated with venous thrombosis present in the amplified material (see below).

The hybridization conditions are selected to permit discrimination between matched and mismatched oligonucleotides. Hybridization conditions can be chosen to correspond to those known to be suitable in standard procedures for hybridization to filters and then optimized for use with the arrays of the disclosure. For example, conditions suitable for hybridization of one type of target would be adjusted for the use of other targets for the array. In particular, temperature is controlled to substantially eliminate formation of duplexes between sequences other than exactly complementary VT-associated wild-type of mutant sequences. A variety of known hybridization solvents can be employed, the choice being dependent on considerations known to one

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of skill in the art (see U.S. Patent 5,981,185, herein incorporated by reference).

Once the amplified nucleic acid molecules associated with venous thrombosis have been hybridized with the oligonucleotides present in the VT detection array, the presence of the hybridization complex can be analyzed, for example by detecting the  
5 complexes.

Detecting a hybridized complex in an array of oligonucleotide probes has been previously described (see U.S. Patent No. 5,985,567, herein incorporated by reference). In one example, detection includes detecting one or more labels present on the oligonucleotides, the amplified sequences, or both. In particular examples, developing  
10 includes applying a buffer. In one embodiment, the buffer is sodium saline citrate, sodium saline phosphate, tetramethylammonium chloride, sodium saline citrate in ethylenediaminetetra-acetic, sodium saline citrate in sodium dodecyl sulfate, sodium saline phosphate in ethylenediaminetetra-acetic, sodium saline phosphate in sodium dodecyl sulfate, tetramethylammonium chloride in ethylenediaminetetra-acetic,  
15 tetramethylammonium chloride in sodium dodecyl sulfate, or combinations thereof. However, other suitable buffer solutions can also be used.

Detection can further include treating the hybridized complex with a conjugating solution to effect conjugation or coupling of the hybridized complex with the detection label, and treating the conjugated, hybridized complex with a detection reagent. In one  
20 example, the conjugating solution includes streptavidin alkaline phosphatase, avidin alkaline phosphatase, or horseradish peroxidase. Specific, non-limiting examples of conjugating solutions include streptavidin alkaline phosphatase, avidin alkaline phosphatase, or horseradish peroxidase. The conjugated, hybridized complex can be treated with a detection reagent. In one example, the detection reagent includes  
25 enzyme-labeled fluorescence reagents or calorimetric reagents. In one specific non-limiting example, the detection reagent is enzyme-labeled fluorescence reagent (ELF) from Molecular Probes, Inc. (Eugene, OR). The hybridized complex can then be placed on a detection device, such as an ultraviolet (UV) transilluminator (manufactured by UVP, Inc. of Upland, CA). The signal is developed and the increased signal intensity

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can be recorded with a recording device, such as a charge coupled device (CCD) camera (manufactured by Photometrics, Inc. of Tucson, AZ). In particular examples, these steps are not performed when radiolabels are used.

In particular examples, the method further includes quantification, for instance  
5 by determining the amount of hybridization.

### **Kits**

The present disclosure provides for kits that can be used to determine whether a subject, such as an otherwise healthy human subject, is genetically predisposed to  
10 venous thrombosis. Such kits allow one to determine if a subject has one or more genetic mutations or polymorphisms in sequences associated with venous thrombosis, including those listed in Table 1.

The disclosed kits include a binding molecule, such as an oligonucleotide probe that selectively hybridizes to a VT-related molecule (such as a mutant or wild-type  
15 nucleic acid molecule) that is the target of the kit. In one example, the kit includes the oligonucleotide probes shown in SEQ ID NOS: 1-287, or a subset thereof, such as even-numbered SEQ ID NOS: 2-284 and SEQ ID NO: 287 or odd-numbered SEQ ID NOS: 1-285 and SEQ ID NO: 286. In another example, a kit includes at least 20 of the probes shown in SEQ ID NOS: 1-287, such as at least 50, at least 75, at least 100, at least 125,  
20 at least 150, at least 175, at least 200, at least 225, or at least 250 of the probes shown in SEQ ID NOS: 1-287. It is understood that fragments of the full-length probes shown in SEQ ID NOS: 1-287 can also be used, such as fragments that include at least 15 contiguous nucleotides of any of SEQ ID NOS: 1-287, such as at least 16 contiguous nucleotides, such as at least 17 contiguous nucleotides, such as at least 18 contiguous  
25 nucleotides, such as at least 19 contiguous nucleotides, such as at least 20 contiguous nucleotides, such as at least 21 contiguous nucleotides, such as at least 22 contiguous nucleotides, such as at least 23 contiguous nucleotides, or such as at least 24 contiguous nucleotides, of any of SEQ ID NOS: 1-287.

In a particular example, kits include antibodies capable of binding to wild-type

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VT-related proteins or to mutated or polymorphic proteins. Such antibodies have the ability to distinguish between a wild-type and a mutant or polymorphic VT-related protein.

The kit can further include one or more of a buffer solution, a conjugating  
5 solution for developing the signal of interest, or a detection reagent for detecting the signal of interest, each in separate packaging, such as a container. In another example, the kit includes a plurality of VT-related target nucleic acid sequences for hybridization with a VT detection array to serve as positive control. The target nucleic acid sequences can include oligonucleotides such as DNA, RNA, and peptide-nucleic acid, or can  
10 include PCR fragments.

### **Venous Thrombosis Preventative Therapy**

The present disclosure also provides methods of avoiding or reducing the incidence of venous thrombosis in a subject determined to be genetically predisposed to  
15 developing venous thrombosis. For example, if using the screening methods described above a mutation or polymorphism in at least one VT-related molecule in the subject is detected, a treatment is selected to avoid or reduce the incidence of venous thrombosis or to delay the onset of venous thrombosis. The subject then can be treated in accordance with this selection, for example by administration of one or more  
20 anticoagulant agents. In some examples, the treatment selected is specific and tailored for the subject, based on the analysis of that subject's profile for one or more VT-related molecules.

The disclosure is further illustrated by the following non-limiting Examples.  
25

#### **EXAMPLE 1**

##### **Mutations and Polymorphisms Associated with Venous Thrombosis**

Table 1 describes VT-related nucleic acid and protein sequences used to design an array that allows for screening of all currently known 143 venous thrombosis

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associated recurrent mutations and polymorphisms in eight different genes. However, one skilled in the art will appreciate that additional recurrent VT-associated mutations and polymorphisms not currently identified can also be used. For each potential site of mutation/polymorphism, two oligonucleotide probes were designed (see Example 3).

5

**Table 1: Mutations and polymorphisms associated with venous thrombosis.**

<b>Gene</b>	<b>Mutation or polymorphism*</b>
AT III	<u>Type I AT III deficiency</u> : 2770insT, 5311-5320del6bp, 5356-64delCTT, 5381C/T, 5390C/T, 5493A/G, 6490C/T, 9788G/A, 9819C/T, 13342insA, 13380T/C <u>Type II AT III deficiency</u> : <i>RS mutations</i> : 6460A/G, 13262G/A, 13268G/C, 13268G/T, 13295C/T, 13296G/A and 13299C/T <i>HBS mutations</i> : 2484T/A, 2586C/T, 2603C/T, 2604G/A, 2759C/T, 5382G/A <i>PE mutations</i> : 13324C/A, 13328G/A, 13333C/G, 13337C/A, 13338C/T, 13392G/C
Protein C	<u>Type I PC deficiency</u> : 41G/A; 1357C/T; 1381C/T; 3103C/T; 3169T/C; 3217G/T; 3222G/A; 3222G/T; 3359G/A; 3360C/A; 3363/4, insC; 3439C/T; 6128T/C; 6152C/T; 6182C/T; 6216C/T; 6245C/T; 6246G/A; 6265G/C; 6274C/T; 7176G/A; 7253C/T; 8403C/T; 8481A/G; 8485/6 delAC or 8486/7 delCA; 8551C/T; 8559G/A; 8571C/T; 8572G/A; 8589G/A; 8604G/A; 8608C/T; 8631C/T; 8678-80 del3nt; 8689T/C; 8695C/T; 8763G/A; 8857, delG; 8895A/C; 8924C/G <u>Type II PC deficiency</u> : 1387C/T; 1388G/A; 1432C/T; 6218C/T; 6219G/A; 7219C/A; 8470G/A; 8744G/A; 8769C/T; 8790G/A; 8886G/A <u>PC gene polymorphisms</u> : -1654C/T; -1641A/G, -1476A/T
Protein S	<u>Quantitative PS deficiency (type I and type III)</u> : -34, TC (delG); -24, GTG/GAG; 19, GAA/TAA; 26, GAA/GCA; 44, TA (delCTTA); 46, GTT/CTT; intron d, G/A, exon 4 +1; 155, AAG/GAG; 217, AAT/AGT; 238, CAG/TAG; 265, TTT (ins T), 293, TCA/TGA; 295, GGC/GTC; intron j, G/A, exon 10 +5; 349, GAA/AAA; 372, delCTTTT, insAA; intron k, A/G, exon 12 -9; 405, CTA/CCA; 410, CGA/TGA; 431, AA (insA); 465, TGG/TGA; 474, CGT/TGT; 522, CAG/TAG; 534,

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	<p>CTG/CGG; 625, TGT/CGT</p> <p><u>Qualitative PS deficiency (type II):</u> -2, CGT/CTT; 9, AAA/GAA; intron e, G/A, exon 5 +5</p> <p><u>Unknown type of PS deficiency:</u> -25, CT (insT); 467, GTA/GGA; 633, (delAA); 636, TAA/TAT</p> <p><u>PS gene polymorphisms:</u> intron k, C/T, exon 11 +54; 460, TCC/CCC; 626, CCA/CCG; exon 15, C/A 520 nt after the stop codon</p>
Fibrinogen	<p><u><math>\alpha</math> chain:</u> <math>\alpha</math>(16)Arg/Cys; <math>\alpha</math>(16)Arg/His; <math>\alpha</math>(19)Arg/Gly; <math>\alpha</math>(461)Lys/stop; <math>\alpha</math>(554)Arg/Cys</p> <p><u><math>\beta</math> chain:</u> <math>\beta</math>(14)Arg/Cys; <math>\beta</math>(68)Ala/Thr; <math>\beta</math>(255)Arg/Cys</p> <p><u><math>\gamma</math> chain:</u> <math>\gamma</math>(275)Arg/Cys; <math>\gamma</math>(275)Arg/His; <math>\gamma</math>(292)Gly/Val; <math>\gamma</math>(308)Asn/Lys; <math>\gamma</math>(318)Asp/Gly</p> <p><u>Fibrinogen gene polymorphism:</u> Thr312Ala</p>
Factor V	1691G/A; 1628G/A; 4070A/G; 1090A/G; 1091G/C
Prothrombin (Factor II)	20210G/A
MTHFR	677C/T; 1298A/C
ACE	Intron 16, 288 bp insertion/deletion

\*Nucleotide or amino acid number refers to the human sequence, although one skilled in the art can determine the corresponding nucleotide or amino acid for other organisms.

## EXAMPLE 2

### 5 Statistical Analysis in the Prediction of Venous Thrombosis

This example demonstrates that MERT offers a high magnitude clinical validity by assessing 143 alleles simultaneously in identifying individuals at very high risk of developing VT, even if the contribution of each allele to the risk is small and not enough to cause VT.

10 To demonstrate statistically that the disclosed methods can predict a healthy subject's probability of developing venous thrombosis, the following methods were used. The results described below demonstrate that disease prediction for venous thrombosis is greatly improved by considering multiple predisposing genetic factors concurrently. To demonstrate how concurrent screening of multiple venous thrombosis

15 (VT) associated susceptibility gene defects improves the prediction of developing venous thrombosis, likelihood ratios for each VT associated susceptibility gene test were calculated by logistic regression and then the combined likelihood ratio (LR) for the panel of VT associated susceptibility gene tests was calculated simply as the product

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of the likelihood ratios (LRs) of the individual tests assuming each test is independent.

For the calculations, 10 VT associated susceptibility alleles in eight VT associated genes with an established prevalence both in control subjects and unselected VT patients were selected.

- 5           The relevant allele frequencies were derived for AT III, protein C and protein S deficiencies, fibrinogen Thr312Ala, FV Leiden (G1691A), FV G1628A, FV A4070G (R2 allele), prothrombin G20210A, MTHFR C677G, and ACE DD variants using data from previously reported case-control studies conducted in the different ethnic populations regarding VT associated genetic susceptibility (Seligsohn and Lubetsky, *N. Engl. J. Med.* 344:1222-31, 2001; Heijboer *et al. N. Engl. J. Med.* 323:1512-6, 1990; Pabinger *et al., Blood. Coagul. Fibrinolysis* 3:547-53, 1992; Melissari *et al., Blood. Coagul. Fibrinolysis* 3:749-58, 1992; Bombeli *et al. Am. J. Hematol.* 70:126-32, 2002; Salomon *et al., Arterioscler. Thromb. Vasc. Biol.* 19:511-8, 1999; Harper *et al., Br. J. Haematol.* 77:360-364, 1991; Tait *et al. Br. J. Haematol.* 87:106-12, 1994; Arruda *et al., Thromb. Haemost.* 77:818-21, 1997; Junker *et al., Arterioscler. Thromb. Vasc. Biol.* 19:2568-72, 1999; Heller *et al., Circulation.* 108:1362-7, 2003; Jerrard-Dunne *et al. Stroke.* 34:1821-7, 2003; Patel *et al. Thromb. Haemost.* 90:835-8, 2003; Shen *et al. Thromb. Res.* 99:447-52, 2000; Sakata *et al. J. Thromb. Hemost.* 2:528-30, 2004; Lee *et al. Ann. Acad. Med. Singapore.* 31:761-4, 2002; Liu *et al. Thromb. Haemost.* 71:416-9, 15 1994; Suehisa *et al. Blood. Coagul. Fibrinolysis.* 12:95-9, 2001; Chen *et al. Ann. Hematol.* 82:114-7, 2003; Ho *et al. Am. J. Hematol.* 63:74-8, 2000; Miletich *et al., N. Engl. J. Med.* 317:991-6, 1987; Horellou *et al., BMJ* 289:1285-1287, 1984; Gladson *et al., Thromb. Haemost.* 59:18-22, 1988; Tait *et al., Thromb. Haemost.* 73:87-93, 1995; Dykes *et al., Br. J. Haematol.* 113:636-41; 2001; Carter *et al., Blood.* 96:1177-9, 2000; 20 Liu Y *et al., J. Med. Genet.* 38:31-5, 2001; De Stefano *et al. Semin. Thromb. Hemost.* 24:367-79, 1998; Benson *et al. Thromb. Haemost.* 86:1188-92, 2001; Ehrenforth *et al. Arterioscler. Thromb. Vasc. Biol.* 19:276-80, 1999; Leroyer *et al. Thromb. Haemost.* 80:49-51, 1998; Brown *et al. Br. J. Haematol.* 98:907-9, 1997; Arruda *et al. Thromb. Haemost.* 78:1430-3, 1997; de Moerloose *et al., Thromb. Haemost.* 80:239-41, 1998;

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- Dowling *et al.* *J. Thromb. Hemost.* 1:80-7, 2003; Rees *et al.* *Br. J. Haematol.* 105:564-6, 1999; Helley *et al.* *Hum. Genet.* 100:245-8, 1997; Le *et al.* *Clin. Genet.* 57:296-303, 2000; Lu *et al.*, *Thromb. Res* 107:7-12, 2002; Dilley *et al.*, *Am. J. Epidemiol.* 147:30-5, 1998; Faisel *et al.*, *Eur. J. Hum. Genet.* 12:187-91, 2004; Dogulu *et al.*, *Thromb. Res.* 5 111:389-95, 2003; Hiyoshi *et al.* *Thromb. Haemost.* 80:705-6, 1998; Watanabe *et al.* *Thromb. Haemost.* 86:1594-5, 2001; Alhenc-Gelas *et al.* *Thromb. Haemost.* 81:193-97, 1999; Poort *et al.* *Blood* 88:3698-703, 1996; Hillarp *et al.* *Thromb. Haemost.* 78:990-2, 1997; Ferraresi *et al.* *Arterioscler. Thromb. Vasc. Biol.* 17:2418-22, 1997; Corral *et al.* *Br. J. Haematol.* 99:304-307, 1997; Hainaut *et al.* *Acta Clin Belg* 53:344-348, 1998; 10 Cumming *et al.* *Br. J. Haematol.* 98:353-355, 1997; Souto *et al.* *Thromb. Haemost.* 80:366-9, 1998; Eichinger *et al.* *Thromb. Haemost.* 81:14-7, 1999; Tosetto *et al.* *Thromb. Haemost.* 82:1395-98, 1999; Ridker *et al.* *Circulation.* 99:999-1004, 1999; Margaglione *et al.*, *Thromb. Haemost.* 79:907-11, 1998; Dilley *et al.*, *J. Lab. Clin. Med.* 132:452-5, 1998; Howard *et al.* *Blood* 91:1092, 1998; Zheng *et al.*, *Br. J. Haematol.* 15 109:870-4, 2000; Lin *et al.*, *Thromb. Res.* 97:89-94, 2000; Fatini *et al.*, *Eur. J. Clin. Invest.* 33:642-7, 2003; and Hooper *et al.*, *Am. J. Hematol.* 70:1-8, 2002) (Table 2).

**Table 2: Frequency of inherited thrombophilias among control subjects and unselected patients with VT**

	Control subjects		Unselected patients	
	Total Screened	Number tested positive	Total Screened	Number tested positive
<b>Antithrombin III deficiency</b>	15,610	33 (0.2%)	3,509	122 (3.5%)
<b>Protein C deficiency</b>	21,011	45 (0.2%)	3,557	193 (5.4%)
<b>Protein S deficiency</b>	5,212	28 (0.5 %)	3,332	189 (5.7%)
<b>Fibrinogen gene Thr312Ala polymorphism</b>	250 <sup>I</sup> 402 <sup>II</sup>	101(40.4%) 270(67.2%)	218	110 (50.4%)
<b>FV gene G1691A (Leiden) polymorphism</b>	20,313 <sup>I</sup> 8,211 <sup>III</sup>	1,091 (5.4%) 54 (0.7%)	3,651	644 (17.6 %)
<b>FV gene G1628A polymorphism</b>	245 <sup>I</sup> 505 <sup>II</sup> 245 <sup>IV</sup>	22 (9%) 360 (72%) 132 (54%)	133 156	26 (20%) 145 (93%)
<b>FV gene A4070G (R2 allele) polymorphism</b>	394 <sup>I</sup> 2,029 <sup>V</sup>	45 (11.4%) 114 (5.6%)	205	38 (18.5%)
<b>Prothrombin gene G20210A polymorphism</b>	7,110 <sup>I</sup> 2,299 <sup>III</sup>	188 (2.6%) 1 (0.04%)	4,312	222 (5.1 %)
<b>MTHFR gene C677T polymorphism (TT)</b>	1,222 <sup>I</sup> 372 <sup>II</sup>	146 (11.9%) 67 (18%)	328	62 (18.9%)
<b>ACE gene DD genotype</b>	378 <sup>I</sup> 370 <sup>VI</sup>	101 (26.7%) 80 (21.6%)	208 184	99 (48%) 71 (38.6%)

<sup>I</sup> Caucasian subjects

<sup>II</sup> Asian subjects

5 <sup>III</sup> Non-European subjects from Africa, North America, Asia, Australasia, Latin America and Middle East and Inuit subjects.

<sup>IV</sup> African subjects

<sup>V</sup> Non-European subjects from North America, Latin America, Asia and Pacific Islands.

<sup>VI</sup> African-American subjects

10

The LR calculations were performed by logistic regression. By treating the data retrieved from the previously reported case control studies regarding VT genetic susceptibility in different ethnic populations as a valid estimate of the risk odds ratio, LR for each allele positive test was calculated by exponentiation of the result obtained as previously described (Albert, *Clin. Chem.*, 28:1113-9, 1982; McCullagh and Nelder, *Chapman and Hall, London*, 1989; Yang *et al.*, *Am. J. Hum. Genet.*, 72:636-49, 2003).

15

The posterior probability of venous thrombosis (the probability of developing venous thrombosis) was determined for the individuals with allele-positive test results

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for each genetic test (also known as positive predictive value of each genetic test).

Calculated likelihood ratios and positive predictive values for each venous thrombosis associated susceptibility gene test were demonstrated in Table 3.

5 **Table 3. Likelihood ratios and Positive predictive values of single susceptibility genes and multiple genetic screening with MERT for developing VT in healthy subjects**

Single susceptibility test analysis	Likelihood Ratio	Posterior probability of developing VT
Antithrombin III deficiency	16.4	1.6%
Protein C deficiency	25.3	2.5%
Protein S deficiency	10.6	1.0%
Fibrinogen Thr312Ala polymorphism	1.25 †	0.12%
Factor V gene		
G1691A (Leiden) polymorphism	3.28 †	0.33%
G1628A polymorphism	2.18 †	0.22%
	1.3 ‡	0.13%
A4070G polymorphism (R2 allele)	1.62 †	0.16%
Prothrombin gene G20210A polymorphism	1.95 †	0.2%
MTHFR gene C677T polymorphism (TT)	1.58 †	0.16%
ACE DD genotype	1.78 †	0.18%
	1.78 *	0.18%
<b>Concurrent screening of 8 genes with MERT</b>	349250.7 †	99.7%
	5717.6 ‡	85.1%
	7828.7 *	88.7%

† Caucasian populations; ‡ Asian populations; \*African populations

10 Then, assuming that the effect of each of the genetic defects in the eight different genes is independent and that all interactive effects are purely multiplicative, the LR was calculated for the panel of ten VT associated genetic susceptibility tests as the product of the likelihood ratios of the individual test results.

15 As shown in Table 3, whereas each genetic test provides limited predictive information about the probability of developing venous thrombosis (the posterior probabilities of disease range from 0.12% to 2.5% for each test alone), the posterior probability of venous thrombosis occurring increases to 99.7% when estimated with unselected patients for Caucasians and 85.1% for Asians and 88.7% for African

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populations by using the disclosed methods, an increase of > 30-fold.

### EXAMPLE 3

#### Array for Detecting Susceptibility to Venous Thrombosis

5 For each potential site of mutation/polymorphism (Table 1), two oligonucleotide probes were designed (SEQ ID NOS 1-287). The first is complementary to the wild type sequence (odd numbers of SEQ ID NOS: 1-285 and SEQ ID NO: 286) and the second is complementary to the mutated sequence (even numbers of SEQ ID NOS: 2-284 and SEQ ID NO: 287). For example, SEQ ID NO: 1 is complementary to a wild-  
10 type ATIII sequence, and SEQ ID NO: 2 is complementary to a mutant ATIII sequence, which can be used to detect the presence of a "T" insertion at nucleotide 2770. The disclosed oligonucleotide probes can further include one or more detectable labels, to permit detection of hybridization signals between the probe and a target sequence.

15 Compilation of "loss" and "gain" of hybridization signals will reveal the genetic status of the individual with respect to the 143 known VT-associated recurrent defects.

### EXAMPLE 4

#### Nucleic Acid-Based Analysis

20 The VT-related nucleic acid molecules provided herein can be used in methods of genetic testing for predisposition to venous thrombosis owing to VT-related nucleic acid molecule polymorphism/mutation in comparison to a wild-type nucleic acid molecule. For such procedures, a biological sample of the subject is assayed for a polymorphism or mutation (or both) in a VT-related nucleic acid molecule, such as those listed in Table 1. Suitable biological samples include samples containing genomic  
25 DNA or RNA (including mRNA) obtained from cells of a subject, such as those present in peripheral blood, urine, saliva, tissue biopsy, surgical specimen, amniocentesis samples and autopsy material.

The detection in the biological sample of a polymorphism/mutation in one or more VT-related nucleic acid molecules, such as those listed in Table 1, can be achieved

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by methods such as hybridization using allele specific oligonucleotides (ASOs) (Wallace *et al.*, *CSHL Symp. Quant. Biol.* 51:257-61, 1986), direct DNA sequencing (Church and Gilbert, *Proc. Natl. Acad. Sci. USA* 81:1991-1995, 1988), the use of restriction enzymes (Flavell *et al.*, *Cell* 15:25, 1978; Geever *et al.*, 1981), discrimination  
5 on the basis of electrophoretic mobility in gels with denaturing reagent (Myers and Maniatis, Cold Spring Harbor Symp. Quant. Biol. 51:275-84, 1986), RNase protection (Myers *et al.*, *Science* 230:1242, 1985), chemical cleavage (Cotton *et al.*, *Proc. Natl. Acad. Sci. USA* 85:4397-401, 1985), and the ligase-mediated detection procedure (Landegren *et al.*, *Science* 241:1077, 1988).

10 Oligonucleotides specific to wild-type or mutated VT-related sequences can be chemically synthesized using commercially available machines. These oligonucleotides can then be labeled, for example with radioactive isotopes (such as <sup>32</sup>P) or with non-radioactive labels such as biotin (Ward and Langer *et al.*, *Proc. Natl. Acad. Sci. USA* 78:6633-6657, 1981) or a fluorophore, and hybridized to individual DNA samples  
15 immobilized on membranes or other solid supports by dot-blot or transfer from gels after electrophoresis. These specific sequences are visualized, for example by methods such as autoradiography or fluorometric (Landegren *et al.*, *Science* 242:229-237, 1989) or colorimetric reactions (Gebeyehu *et al.*, *Nucleic Acids Res.* 15:4513-4534, 1987). Using an ASO specific for a wild-type allele, the absence of hybridization would  
20 indicate a mutation or polymorphism in the particular region of the gene. In contrast, if an ASO specific for a mutant allele hybridizes to a clinical sample then that would indicate the presence of a mutation or polymorphism in the region defined by the ASO.

## EXAMPLE 5

### 25 Protein-Based Analysis

This example describes methods that can be used to detect defects in an amount of a VT-related protein, or to detect changes in the amino acid sequence itself. VT-related protein sequences can be used in methods of genetic testing for predisposition to venous thrombosis owing to VT-related protein polymorphism or mutation (or both) in

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comparison to a wild-type protein. For such procedures, a biological sample of the subject is assayed for a polymorphism or mutation in a VT-related protein, such as those listed in Table 1. Suitable biological samples include samples containing protein obtained from cells of a subject, such as those present in peripheral blood, urine, saliva,  
5 tissue biopsy, surgical specimen, amniocentesis samples and autopsy material.

A decrease in the amount of one or more VT-related proteins in a subject can indicate that the subject has an increased susceptibility to developing VT. Similarly, the presence of one or more mutations or polymorphisms in a VT-related protein in comparison to a wild-type protein can indicate that the subject has an increased  
10 susceptibility to developing VT.

The determination of reduced VT-related protein levels, in comparison to such expression in a normal subject (such as a subject not predisposed to developing VT), is an alternative or supplemental approach to the direct determination of the presence of VT-related nucleic acid mutations or polymorphisms by the methods outlined above.  
15 The availability of antibodies specific to particular VT-related protein(s) will facilitate the detection and quantitation of cellular VT-related protein(s) by one of a number of immunoassay methods which are well known in the art, such as those presented in Harlow and Lane (*Antibodies, A Laboratory Manual*, CSHL, New York, 1988).  
Methods of constructing such antibodies are known in the art.

20 The determination of the presence of one or more mutations or polymorphisms in a VT-related protein, in comparison to a wild-type VT-related protein, is another alternative or supplemental approach to the direct determination of the presence of VT-related nucleic acid mutations or polymorphisms by the methods outlined above. Antibodies that can distinguish between a mutant or polymorphic protein and a wild-  
25 type protein can be prepared using methods known in the art.

Any standard immunoassay format (such as ELISA, Western blot, or RIA assay) can be used to measure VT-related polypeptide or protein levels, and to detect mutations or polymorphisms in VT-related proteins. A comparison to wild-type (normal) VT-related protein levels and a decrease in VT-related polypeptide levels is indicative of

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predisposition to developing VT. Similarly, the presence of one or more mutant or polymorphic VT-related proteins is indicative of predisposition to developing VT. Immunohistochemical techniques can also be utilized for VT-related polypeptide or protein detection and quantification. For example, a tissue sample can be obtained from  
5 a subject, and a section stained for the presence of a wild-type or polymorphic or mutant VT-related protein using the appropriate VT-related protein specific binding agents and any standard detection system (such as one that includes a secondary antibody conjugated to horseradish peroxidase). General guidance regarding such techniques can be found in Bancroft and Stevens (*Theory and Practice of Histological Techniques*,  
10 Churchill Livingstone, 1982) and Ausubel *et al.* (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1998).

For the purposes of quantitating a VT-related protein, a biological sample of the subject, which sample includes cellular proteins, can be used. Quantitation of a VT-related protein can be achieved by immunoassay and the amount compared to levels of  
15 the protein found in cells from a subject not genetically predisposed to developing VT. A significant decrease in the amount of one or more VT-related proteins in the cells of a subject compared to the amount of the same VT-related protein found in normal human cells is usually about a 30% or greater difference. Substantial underexpression of one or more VT-related protein(s) can be indicative of a genetic predisposition to developing  
20 VT.

## EXAMPLE 6

### Kits

Kits are provided to determine whether a subject has one or more  
25 polymorphisms or mutations in a VT-related nucleic acid sequence (such as kits containing VT detection arrays). Kits are also provided that contain the reagents need to detect hybridization complexes formed between oligonucleotides on an array and VT-related nucleic acids amplified from a subject. These kits can each include instructions, for instance instructions that provide calibration curves or charts to compare with the

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determined (such as experimentally measured) values.

In one example, the kit includes primers capable of amplifying VT-related nucleic acid molecules, such as those listed in Table 1. In particular examples, the primers are provided suspended in an aqueous solution or as a freeze-dried or lyophilized powder. The container(s) in which the primers are supplied can be any conventional container that is capable of holding the supplied form, for instance, microfuge tubes, ampoules, or bottles. In some applications, pairs of primers are provided in pre-measured single use amounts in individual, typically disposable, tubes, or equivalent containers.

The amount of each primer supplied in the kit can be any amount, depending for instance on the market to which the product is directed. For instance, if the kit is adapted for research or clinical use, the amount of each oligonucleotide primer provided likely would be an amount sufficient to prime several *in vitro* amplification reactions. Those of ordinary skill in the art know the amount of oligonucleotide primer that is appropriate for use in a single amplification reaction. General guidelines may for instance be found in Innis *et al.* (*PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc., San Diego, CA, 1990), Sambrook *et al.* (*In Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989), and Ausubel *et al.* (*In Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1998).

In particular examples, a kit includes an array with oligonucleotides that recognize wild-type, mutant or polymorphic VT-related sequences, such as those listed in Table 1. The array can include other oligonucleotides, for example to serve as negative or positive controls. The oligonucleotides that recognize the wild-type and mutant sequences can be on the same array, or on different arrays. A particular array is disclosed in Example 3. For example, the kit can include the oligonucleotides shown in SEQ ID NOS: 1-287, or subsets thereof, such as at least 10 of the oligonucleotides shown in SEQ ID NOS: 1-287, for example at least 20, at least 50, at least 100, at least 143, or even at least 250 of the oligonucleotides shown in SEQ ID NOS: 1-287. In a particular example, an array includes the odd-numbered SEQ ID NOS: 1-285 (*i.e.* SEQ

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ID NOS: 1, 3, 5, 7, etc.) and in some examples also SEQ ID NO: 286, or the even-numbered SEQ ID NOS: 2-284 (*i.e.* SEQ ID NOS: 2, 4, 6, 8, etc.) and in some examples also SEQ ID NO: 287. However, both such arrays can be included in a single kit.

In some examples, kits further include the reagents necessary to carry out hybridization and detection reactions, including, for instance appropriate buffers. Written instructions can also be included.

Kits are also provided for the detection of VT-related protein expression, for instance under expression of a protein encoded for by a nucleic acid molecule listed in Table 1. Such kits include one or more wild-type or mutant AT III, protein C, protein S, fibrinogen, factor V (FV), prothrombin (factor II), MTHFR and ACE proteins (full-length, fragments, or fusions) or specific binding agent (such as a polyclonal or monoclonal antibody or antibody fragment), and can include at least one control. The VT-related protein specific binding agent and control can be contained in separate containers. The kits can also include a means for detecting VT-related protein:agent complexes, for instance the agent may be detectably labeled. If the detectable agent is not labeled, it can be detected by second antibodies or protein A, for example, either of both of which also can be provided in some kits in one or more separate containers. Such techniques are well known.

Additional components in some kits include instructions for carrying out the assay. Instructions permit the tester to determine whether VT-linked expression levels are reduced in comparison to a control sample. Reaction vessels and auxiliary reagents such as chromogens, buffers, enzymes, etc. can also be included in the kits.

In view of the many possible embodiments to which the principles of our invention may be applied, it should be recognized that the illustrated embodiment is only a preferred example of the invention and should not be taken as a limitation on the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

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We claim:

1. A method of detecting genetic predisposition to venous thrombosis (VT) in a subject, comprising:
  - determining whether the subject has one or more mutations or polymorphisms in
  - 5 at least eight VT-related molecules, wherein the at least eight venous thrombosis molecules comprise antithrombin III (AT III), protein C, protein S, fibrinogen, factor V (FV), prothrombin (factor II), methylenetetrahydrofolate reductase (MTHFR) and angiotensin 1-converting enzyme (ACE), and wherein the presence of one or more mutations or polymorphisms indicates that the subject has a genetic predisposition for
  - 10 venous thrombosis.
2. The method of claim 1, wherein the one or more mutations or polymorphisms comprise one or more mutations or polymorphisms listed in Table 1.
- 15 3. The method of claim 1, wherein the method comprises determining whether the subject has one or more mutations or polymorphisms in at least 10 of the mutations or polymorphisms listed in Table 1.
4. The method of claim 1, wherein the method comprises determining whether the
- 20 subject has one or more mutations or polymorphisms in at least 50 of the mutations or polymorphisms listed in Table 1.
5. The method of claim 1, wherein the method comprises determining whether the subject has one or more mutations or polymorphisms in at least 143 of the mutations or
- 25 polymorphisms listed in Table 1.
6. The method of claim 1, wherein the method comprises determining whether the subject has one or more mutations or polymorphisms in no more than 10 of the mutations or polymorphisms listed in Table 1.

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7. The method of claim 2, wherein the one or more mutations or polymorphisms comprise AT III deficiency, PC deficiency, PS deficiency, fibrinogen Thr312Ala polymorphism, FV Leiden (G1691A) polymorphism, FV G1628 polymorphism, FV  
5 A4070G polymorphism, prothrombin G20210A polymorphism, MTHFR C677T and ACE intron 16, 288 bp insertion/deletion polymorphism.
8. The method of claim 1, wherein the method provides a probability of developing VT  
10 of at least 98% in Caucasians, at least 85% in Asians, and at least 87% in Africans.
9. The method of claim 1, wherein the method comprises determining whether the subject has one or more mutations or polymorphisms in at least eight VT-related molecules.
- 15 10. The method of claim 1, wherein the at least eight VT-related molecules comprise nucleic acid molecules.
11. The method of claim 10, wherein the nucleic acid molecules are amplified from the subject, thereby generating amplification products, and wherein the amplification  
20 products are hybridized with oligonucleotide probes that detect the one or more mutations or polymorphisms.
12. The method of claim 11, wherein hybridizing the oligonucleotides comprises:  
incubating the amplification products with the oligonucleotide probes for a time  
25 sufficient to allow hybridization between the amplification products and oligonucleotide probes, thereby forming amplification products: oligonucleotide probe complexes; and  
analyzing the amplification products: oligonucleotide probe complexes to determine if the amplification products comprise one or more mutations or polymorphisms in the VT-related nucleic acids, wherein the presence of one or more

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mutations or polymorphisms indicates that the subject has a genetic predisposition for VT.

13. The method of claim 12, wherein analyzing the amplification  
5 products:oligonucleotide probe complexes comprises determining an amount of nucleic acid hybridization, and wherein a greater amount of hybridization to one or more of the mutated sequences, as compared to an amount of hybridization to a corresponding wild-type sequence, indicates that the subject has a genetic predisposition for VT.
- 10 14. The method of claim 12, wherein analyzing the amplification products:oligonucleotide probe complexes includes detecting and quantifying the complexes.
- 15 15. The method of claim 11, wherein the oligonucleotide probes are present on an array substrate.
16. The method of claim 15, wherein the array further comprises oligonucleotide probes complementary to wild-type VT-related nucleic acid molecules.
- 20 17. The method of claim 16, wherein the wild-type VT-related nucleic acid molecules comprise oligonucleotide probes complementary to wild-type AT III, wild-type protein C, wild-type protein S, wild-type fibrinogen, wild-type factor V, wild-type factor II, wild-type MTHFR and wild-type ACE nucleic acid sequences.
- 25 18. The method of claim 1, wherein the at least eight VT-related molecules consist of sequences from AT III, protein C, protein S, fibrinogen, factor V, factor II, , MTHFR and ACE.
19. The method of claim 1, wherein the subject is in a group potentially at risk of

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developing a venous thrombosis.

20. The method of claim 19, wherein the subject is pregnant, is in puerperium, is using oral contraceptives or hormone replacement therapy, has previous thrombosis history,  
5 has or will undergo prolonged immobilization, has a myeloproliferative disorder, has a malignancy, has or will undergo surgery, has a bone fracture, is of advanced age, has antiphospholipid antibodies, or combinations thereof.

21. The method of claim 11, wherein the nucleic acid molecules obtained from the  
10 subject are obtained from serum.

22. A method of detecting genetic predisposition to VT in a subject, comprising:  
applying amplification products to an array, wherein the array comprises  
oligonucleotide probes complementary to mutated AT III, mutated protein C, mutated  
15 protein S, mutated fibrinogen, mutated factor V, mutated factor II, mutated MTHFR and mutated ACE sequences, and wherein the amplification products comprise nucleic acid sequences from AT III, protein C, protein S, fibrinogen, factor V, factor II, MTHFR and ACE, obtained from the subject;  
incubating the amplification products with the array for a time sufficient to allow  
20 hybridization between the amplification products and oligonucleotide probes, thereby forming amplification products: oligonucleotide probe complexes; and  
analyzing the amplification products: oligonucleotide probe complexes to determine if the amplification products comprise one or more mutations or polymorphisms in the AT III, protein C, protein S, fibrinogen, factor V, factor II,  
25 MTHFR or ACE sequences, wherein the presence of one or more mutations or polymorphisms indicates that the subject has a genetic predisposition for VT.

23. A method of selecting a venous thrombosis (VT) therapy, comprising:  
detecting a mutation or polymorphism in at least one VT-related molecule of a

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subject, using the method of claim 1; and

if such mutation or polymorphism is identified, selecting a treatment to avoid or reduce VT, or to delay the onset of VT.

5 24. The method of claim 23, further comprising administering the selected treatment to the subject.

25. The method of claim 24, wherein the selected treatment comprises treating the subject with an anticoagulant agent.

10

26. An array comprising oligonucleotide probes complementary to wild-type gene sequences, mutated gene sequences, or both, wherein the gene sequences comprise coding or non-coding sequences from AT III, protein C, protein S, fibrinogen, factor V, factor II, MTHFR and ACE genes.

15

27. The array of claim 26, wherein the mutated gene sequences comprise ten or more mutations or polymorphisms listed in Table 1.

20 28. The array of claim 27, wherein the mutated gene sequences consist essentially of the mutations or polymorphisms listed in Table 1.

29. A method of detecting a genetic predisposition to venous thrombosis (VT) in a subject, comprising:

25 applying amplification products to the array of claim 13, wherein the amplification products comprise amplified nucleic acids obtained from the subject, wherein the nucleic acids comprise coding or non-coding sequences from AT III, protein C, protein S, fibrinogen, factor V, factor II, , MTHFR and ACE.

incubating the amplification products with the array for a time sufficient to allow hybridization between the amplification products and oligonucleotide probes, thereby

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forming amplification products: oligonucleotide probe complexes; and  
analyzing the amplification products: oligonucleotide probe complexes to  
determine if the amplification products comprise one or more mutations or  
polymorphisms in the AT III, protein C, protein S, fibrinogen, factor V, factor II,  
5 MTHFR, or ACE genes, wherein the presence of one or more mutations or  
polymorphisms indicates that the subject has a genetic predisposition for VT.

30. A kit for detecting a genetic predisposition to venous thrombosis (VT) in a subject,  
comprising:

10 a solid phase nucleic acid array comprising a plurality of oligonucleotide probes  
chemically linked to a solid polymeric support surface in a predetermined pattern,  
wherein the oligonucleotide probes are capable of hybridizing under stringent conditions  
to one or more nucleic acid molecules having VT-related mutations or polymorphisms  
in AT III, protein C, protein S, fibrinogen, factor V, factor II, MTHFR and ACE genes.

15

31. The kit of claim 30, wherein the oligonucleotides comprise SEQ ID NOS: 1-287.

32. The kit of claim 30, further comprising primers for amplifying nucleic acid  
molecules obtained from the subject to obtain amplification products, in separate  
20 packaging, wherein the amplification products comprise sequences from AT III, protein  
C, protein S, fibrinogen, factor V, factor II, MTHFR, and ACE genes;

33. The kit of claim 30, further comprising an amplification enzyme, in separate  
packaging.

25

34. The kit of claim 30, further comprising a buffer solution, in separate packaging.

35. The kit of claim 30, wherein the array further comprises oligonucleotides capable of  
hybridizing under stringent conditions to a wild-type AT III, wild-type protein C, wild-

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type protein S, wild-type fibrinogen, wild-type factor V, wild-type factor II, wild-type MTHFR, and wild-type ACE.

## SEQUENCE LISTING

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US2005/001419

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**  
Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)  
EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>ERALI M ET AL: "EVALUATION OF ELECTRONIC MICROARRAYS FOR GENOTYPING FACTOR V, FACTOR II, AND MTHFR" CLINICAL CHEMISTRY, AMERICAN ASSOCIATION FOR CLINICAL CHEMISTRY. WINSTON, US, vol. 49, no. 5, May 2003 (2003-05), pages 732-739, XP001156163 ISSN: 0009-9147 abstract</p> <p style="text-align: center;">----- -/--</p>	<p>1-7, 10-12, 15-18, 22, 26-35</p>

Further documents are listed in the continuation of box C.       Patent family members are listed in annex.

° Special categories of cited documents :

<p>*A* document defining the general state of the art which is not considered to be of particular relevance</p> <p>*E* earlier document but published on or after the international filing date</p> <p>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>*O* document referring to an oral disclosure, use, exhibition or other means</p> <p>*P* document published prior to the international filing date but later than the priority date claimed</p>	<p>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>*&amp;* document member of the same patent family</p>
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Date of the actual completion of the international search  <b>13 May 2005</b>	Date of mailing of the international search report  <b>06/06/2005</b>
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Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer  <b>Costa Roldán, N</b>
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## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US2005/001419

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	POLLAK E S ET AL: "MICROARRAY-BASED GENETIC ANALYSES FOR STUDYING SUSCEPTIBILITY TO ARTERIAL AND VENOUS THROMBOTIC DISORDERS" ITALIAN HEART JOURNAL, CEPI, CENTRO EDITORIALE PUBBLICITARIO ITALIANO, ROME, IT, vol. 2, no. 8, August 2001 (2001-08), pages 568-572, XP009021015 ISSN: 1129-471X abstract	1-7, 10-12, 15-18, 22,26-35
X	----- ZHONG XIAO-BO ET AL: "Single-nucleotide polymorphism genotyping on optical thin-film biosensor chips." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA. 30 SEP 2003, vol. 100, no. 20, 30 September 2003 (2003-09-30), pages 11559-11564, XP002328069 ISSN: 0027-8424 abstract	1-7, 10-12, 15-18, 22,26-35
X	----- GONZÁLEZ-ORDÓN&TILD ET AL: "Genetic determinants of the venous thromboembolism" QUIMICA CLINICA 2003 SPAIN, vol. 22, no. 5, 2003, pages 367-381, XP001205733 ISSN: 1139-2436 abstract; tables 1-3 page 376	1-14, 18-21
X	----- BUCHHOLZ TINA ET AL: "Inherited thrombophilia: Impact on human reproduction." AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY, vol. 50, no. 1, July 2003 (2003-07), pages 20-32, XP009047496 ISSN: 1046-7408 page 20; table 1 abstract	1-14, 18-21
X	----- US 2003/099958 A1 (MCCARTHY JEANETTE) 29 May 2003 (2003-05-29) abstract; claims 8,26,28,30,32,34,37-43,53-56,65 ----- -/--	1-14, 18-25

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US2005/001419

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FRANCO R F ET AL: "Genetic variations of the hemostatic system as risk factors for venous and arterial thrombotic disease." CURRENT GENOMICS, vol. 4, no. 4, May 2003 (2003-05), pages 309-336, XP009047618 ISSN: 1389-2029 abstract	1-14
X	LI Y-H ET AL: "Genetic risk factors of vascular thrombotic disease in Taiwan" ACTA CARDIOLOGICA SINICA 2000 TAIWAN, vol. 16, no. 4, 2000, pages 197-208, XP009047504 ISSN: 1011-6842 abstract	1-14
P,X	DE 102 37 073 A1 (OGHAM GMBH) 19 February 2004 (2004-02-19) abstract; claims 1-13	1-14, 18-21

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US2005/001419

## Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy (claims 1 to 25 and 29)
2.  Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No  
PCT/US2005/001419

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
US 2003099958	A1	29-05-2003	WO	03020118 A2	13-03-2003
DE 10237073	A1	19-02-2004	EP	1398388 A2	17-03-2004