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Development of a three-biosensor panel for the visual detection of thrombophilia-associated mutations

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ABSTRACT

We developed a rapid and low-cost panel of three assays for visual genotyping of the three most common genetic risk factors in thrombophilia, namely, the single-point mutations in the FV (Leiden factor), FII and MTHFR genes. A triplex PCR was developed for simultaneous amplification of three fragments spanning the interrogated loci. Allele discrimination was accomplished by a 5-min primer extension reaction on each locus. Detection of the extension products was performed by means of a dual-allele dipstick-type DNA biosensor. The biosensor is disposable and allows visual detection and confirmation of the genotyping products within 15 min by hybridization without the need for specialized instruments or expensive reagents. The proposed method was evaluated by genotyping 40 samples with a variety of genotypes for all three mutations. The genotyping results were in full concordance with those obtained by restriction fragment length polymorphism analysis and sequencing.

1. Introduction

The term thrombophilia refers to hemostatic multifactorial disorders, inherited and acquired, that affect about 15% of the Caucasian population predisposed to thrombotic phenomena (Walker et al., 2001). The genetic factors include mutations in genes that code for proteins involved in coagulation, fibrinolysis, and homocysteine metabolism (Key and McGlennen, 2002). A single-point mutation in the coagulation factor V gene, FV G1691A or FV Leiden is the most common inherited risk factor for thrombophilia (Bertina, 1997). Another highly characterized variation, the FII G20210A mutation in the 3′-untranslated region of the prothrombin gene, is found in fewer cases (Poort et al., 1996). More recently, a common variation in the methylenetetrahydrofolate reductase (MTHFR) gene, a C to T transition at nucleotide 677, has been extensively investigated as an independent risk factor (Frosst et al., 1995).

Molecular analysis of single-nucleotide polymorphisms (SNPs) such as FV, FII and MTHFR provides a convenient method for assessing the contribution of individual genetics to thrombosis (Evans and Lee-Tataseo, 2002). For this reason, over the past years, considerable effort has been devoted in the development of accurate, rapid and cost-effective genotyping methods (Hessner et al., 1999; Hunault et al., 1999; Huber et al., 2000; Sanders, 2000; Endler et al., 2001; Patnaik et al., 2004; Cooper and Rezende, 2007; Seipp et al., 2008; Bortolin, 2009). These methods include restriction fragment length polymorphism (Huber et al., 2000), allele-specific PCR (Hessner et al., 1999), PCR in combination with mass spectrometry (Endler et al., 2001) and fluorescence resonance energy transfer (Sanders, 2000), high-resolution amplicon melting (Seipp et al., 2008), the invader assay (Patnaik et al., 2004) and a microparticle immunoassay (Hunault et al., 1999). A high-throughput method was also developed involving PCR, primer extension reaction, capture of the extension products on a bead array, and fluorometric detection using flow-cytometry (Bortolin, 2009).

In recent years, there has been a strong interest toward the development of DNA biosensors for simple and low-cost genotyping assays suitable for the small molecular diagnosis laboratory or point-of-care testing. To this end, an electrochemical DNA biosensor for the detection of factor V Leiden mutation was reported (Ozsoz et al., 2003), in which the target DNA (PCR product) is immobilized on the surface of a pencil graphite electrode and hybridized with oligonucleotide probes conjugated to gold nanoparticles. The hybrids were detected by differential pulse voltammetry. Several other DNA biosensors have been reported but not yet applied to the molecular investigation of thrombophilia-related mutations

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(Scarano et al., 2010; Lucarelli et al., 2008; Cosnier and Mailley, 2008).

The aim of the present work was to develop a simple, rapid and low-cost panel of three assays for visual genotyping of the three most common genetic risk factors for thrombophilia, simultaneously, using dipstick-type DNA biosensors. The assay involves one triplex PCR for all mutations, one primer extension (PEXT) reaction for each mutation and visual detection of the products by the DNA biosensor (one sensor per mutation). The allele-specific primers are labelled with either biotin or fluorescein. An oligonucleotide probe is also included in the PEXT reaction mixture, which is complementary to the extended segment of the PEXT primers and carries a poly(dA) tail. Extension occurs only if the primer has a perfectly complementary 3′-end with the target sequence. In this case, the probe hybridizes to the extended segment of the primer, thus giving high specificity to the detection. The PEXT reaction products are captured on the two test zones of the biosensor by immobilized anti-fluorescein antibody and streptavidin. Gold nanoparticles that carry poly(dT)-strands are used as reporters. Visual detection eliminates the need for specialized equipment and highly qualified technical personnel.

2. Materials and methods

2.1. Instrumentation

Polymerase chain reaction (PCR) and primer extension reaction (PEXT) were performed in the Hybaid PCR Sprint thermal cycler (Middlesex, UK) and in the Eppendorf Mastercycler (Hamburg, Germany), respectively. A digital camera, Kodak DC 120 and the Gel Analyzer software for DNA documentation were purchased from Kodak (New York, NY). The dispensers AirJet AJQ 3000 and BioJet BJQ 3000 as well as the Guillotine cutting module CM 4000 and the Clamshell Laminator were from Biodot (Huntingdon, UK).

2.2. Materials

Nucleospin Blood QuickPure kit was obtained from Schleicher & Schuell (Dassel, Germany). SmarTaq polymerase 7S1 was from HyTest (Turku, Finland). Taq DNA polymerase Vent(exo-) was purchased from Biolabs (Ipswich, MA). Terminal transferase (TdT) and Fx174 DNA/BsuRI marker were from Fermentas (Vilnius, Lithuania). Ultrapure 2′-deoxyribonucleoside 5′-triphosphates (dNTPs) were purchased from Promega (Madison, WI). Streptavidin from Streptomyces avidinii was purchased from Roche Diagnostics (Mannheim, Germany) and rabbit antibody to fluorescein (anti-FITC) was from Biodesign International (Saco, ME). Spin-pure purification columns were obtained from Amersham Bioscience (Piscataway, NJ). Gold nanoparticles (40 nm, 9×10^{10} particles/mL) were purchased from British Biocell International (Cardiff, UK). Nitrocellulose membrane PuraBind A-FB was from Whatman (Kent, UK). The wicking pad, glass-fiber conjugate pad and absorbent pad were from Schleicher & Schuell. The plastic adhesive backing was from Biodot. Oligonucleotides used as primers and probes in the course of this study (Table 1) were synthesized by the Thermo Electron GmbH (Ulm, Germany). A 5′-thiol-modified $(dT)_{30}$ oligo was used for conjugation with gold nanoparticles. A $(dA)_{30}$ oligo was used for the construction of the control zone of the strip. Agarose and ethidium bromide and other common reagents were obtained from Sigma–Aldrich (Streinheim, Germany).

2.3. Tailing of probes with dATP

Oligonucleotide probes used in PEXT reactions were tailed at the 3′-end with dATP using terminal deoxynucleotidyl transferase (TdT). The tailing reaction was carried out in a final volume of 20 μL containing 0.2 M potassium cacodylate, 25 mM Tris, 0.01% Triton X-100, 1 mM $CoCl_2$, 1.5 mM dATP, 300 pmol oligonucleotides and 30 U TdT. The mixture was incubated for 1 h at 37 °C and then the reaction was stopped by adding 2 μL of 0.5 M EDTA.

2.4. Preparation of dry-reagent strip-type DNA biosensor

The dry-reagent strip (4×70 mm) comprised a wicking pad, a glass-fiber conjugate pad, a nitrocellulose diagnostic membrane and an absorbent pad assembled on a plastic adhesive backing (Litos et al., 2007). Oligonucleotide-conjugated gold nanoparticles (prepared according to Litos et al., 2007) were loaded on the conjugate pad of the strip at a density of 3.6 fmol of nanoparticles per 4 mm (strip width) and then allowed to dry at ambient temperature for 15 min. Anti-FITC rabbit antibody, streptavidin and poly(dA) oligonucleotide were immobilized in the first test zone (T1), the second test zone (T2) and the control zone (CZ) of the membrane, respectively. Anti-FITC was diluted in a 2.8 mM PBS solution (pH 7.2), containing 5% sucrose and was loaded at a density of 250 ng per 4 mm. On the other hand, streptavidin was diluted in water con-

Table 1
Oligonucleotide sequences used as primers and probes.

Oligonucleotide	Name	Sequence (5′ → 3′)	Size (mer)	Label
PCR primers				
5′ primer, FVL (319 bp)	up-FVL	GTGCTTAACAAGACCATACTACAGTGACG	29 bp	–
3′ primer, FVL (319 bp)	dwn-FVL	GCCTTCGGCAGTGATGGTACTGAT	24 bp	–
5′ primer, PTH (233 bp)	up-PTH	GGCTTCTACACACATGTGTTCCGCCTG	27 bp	–
3′ primer, PTH (233 bp)	dwn-PTH	AGTAGTATTACTGGCTCTTCCTGAGCCC	28 bp	–
5′ primer, MTHFR (419 bp)	up-MTHFR	GAAGGTGCAAGATCAGAGCCCCCAAA	26 bp	–
3′ primer, MTHFR (419 bp)	dwn-MTHFR	AACTCAGCGAACTCAGCACTCCACCCA	27 bp	–
PEXT primers				
5′ primer, FVL (normal)	N-FVL	GTAAGAGCAGATCCCTGGACAGTCG	25 bp	5′ biotin
5′ primer, FVL (mutant)	M-FVL	GTAAGAGCAGATCCCTGGACAGTCA	26 bp	5′ FITC
5′ primer, PTH (normal)	N-PTH	TGAATAGCACTGGGAGCATTGAGGCTC	27 bp	5′ biotin
5′ primer, PTH (mutant)	M-PTH	TGAATAGCACTGGGAGCATTGAGGCTC	27 bp	5′ FITC
3′ primer, MTHFR (normal)	N-MTHFR	TGAAGGAGAAGGTGTCTGCGGGAGC	25 bp	5′ biotin
3′ primer, MTHFR (mutant)	M-MTHFR	TGAAGGAGAAGGTGTCTGCGGGAGT	25 bp	5′ FITC
Probes				
3′ probe, FLV	probe-FVL	ACACTGGTGCTAAAAAGGACTACTT	25 bp	–
3′ probe, PTH	probe-PTH	GGTCATTGATCAGTTTGGAGAGTAGG	26 bp	–
5′ probe, MTHFR	probe-MTHFR	TCACCTGGATGGGAAAGATCC	21 bp	–
Oligo(dA) for control zone construction	$poly(dA)_{30}$	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	30 bp	–
Oligo(dT) for conjugation to Aequorin	$poly(dT)_{30}$	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	30 bp	5′ thiol

taining 5% sucrose and was loaded at a density of 0.8 mg per 4 mm (width strip). The poly(dA) tailed probe was diluted in water and loaded at a density of 0.6 pmol per 4 mm. The membrane was then dried in an oven for 45 min at 80 °C and the strips were assembled. The strips were stored dry at room temperature.

2.5. Three-biosensor panel for the visual detection of thrombophilia-associated mutations

2.5.1. Triplex PCR

A segment in the FV gene flanking the G1691A mutation, a segment in the FII gene flanking the G20210A mutation and a segment in the MTHFR gene flanking the C677T mutation were amplified in the same vessel by a triplex PCR. The reaction mixture (50-μL) contained 1 U SmartTaq DNA polymerase, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl, 0.1% Tween-20, 2.5 mM $MgCl_2$, 200 μM of each of the dNTPs, 0,2 μM of each primer (3 set of primers) and 4 μL of genomic DNA. Cycling parameters were: initial denaturation at 95 °C for 2 min, 35 cycles at 95 °C (30 s), 55 °C (30 s), 72 °C (30 s) and a final extension step at 72 °C for 5 min.

2.5.2. Primer extension reaction

Aliquots of the PCR product (without purification) were subjected to three separate PEXT reactions (one for each mutation). Each PEXT reaction was performed in a total volume of 10 μL containing 50 mM KCl, 20 mM, $(NH_4)_2SO_4$, 75 mM Tris–HCl, 2 mM $MgCl_2$, 15 mM NaCl, 5 μM of each of the dNTPs, 0.25 U Vent (exo$^-$) DNA polymerase, 4 μL PCR product (100–500 fmol), 0.1 μM of each normal and mutant primer (except for the mutant primer for the C677T mutation which was 0.02 μM) and 0.125 μM of the common oligonucleotide probe complementary to the extended segment of the PEXT primers. The thermal cycling conditions for PEXT reactions were as follows: initial denaturation at 95 °C for 2 min, followed by six cycles of: denaturation at 95 °C for 5 s; primer annealing at 58 °C for 2 s and final extension step at 72 °C for 5 s.

2.5.3. Dual-allele dipstick assay

A 10-μL aliquot of PEXT reaction product was applied onto the conjugate pad above the gold nanoparticles. The bottom part of the strip was then immersed into 300 μL of developing solution containing 10 mM PBS, 4% glycerol and 1% SDS. The visual detection of PEXT products was finished within 10 min.

3. Results and discussion

3.1. Assay principle

The principle of the genotyping reaction (PEXT reaction) is illustrated in Fig. 1. The configuration of the dipstick-type DNA biosensor is presented in Fig. 2. Genomic DNA fragments spanning the three mutations of interest were amplified by a triplex PCR. The products were analyzed by 2.5% agarose gel electrophoresis and ethidium bromide staining. The sizes of PCR products were 319 bp, 233 bp and 419 bp for FV, FII and MTHFR, respectively (Fig. 2). For the genotyping, aliquots of the PCR product (without prior purification or quantification) were subjected to three separate PEXT reactions (one for each locus). Two allele-specific primers were used in each PEXT reaction, which differed in the 3′-end nucleotide and in their 5′-end "tag". The normal primer had a 3′-end nucleotide complementary to the normal allelic variant and was labelled at the 5′-end with biotin, whereas the mutant primer was complementary to the mutant allele and labelled at the 5′-end with the hapten fluorescein. An oligonucleotide probe was also included in the PEXT reaction mixture, which was designed to be complementary to the extended segment of the PEXT primers and carried a poly(dA) tail at the 3′-end. Due to the high accuracy of nucleotide incorporation by Vent (exo-) DNA polymerase, extension occurred only if the primer had a perfectly complementary 3′-end with the target sequence. In this case, the probe hybridized to the extended segment of the primer. If extension did not occur, then there was no hybridization of the probe to the primer.

The PEXT reaction products were visually detected by the dry-reagent dipstick-type biosensor. No purification of the PEXT reaction products is required prior to detection by the DNA biosensor. The biosensor (Fig. 2) comprises two test zones (T1 and T2) and a control zone (C) containing immobilized anti-FITC antibody, streptavidin and poly(dA) probe, respectively. Gold nanoparticles

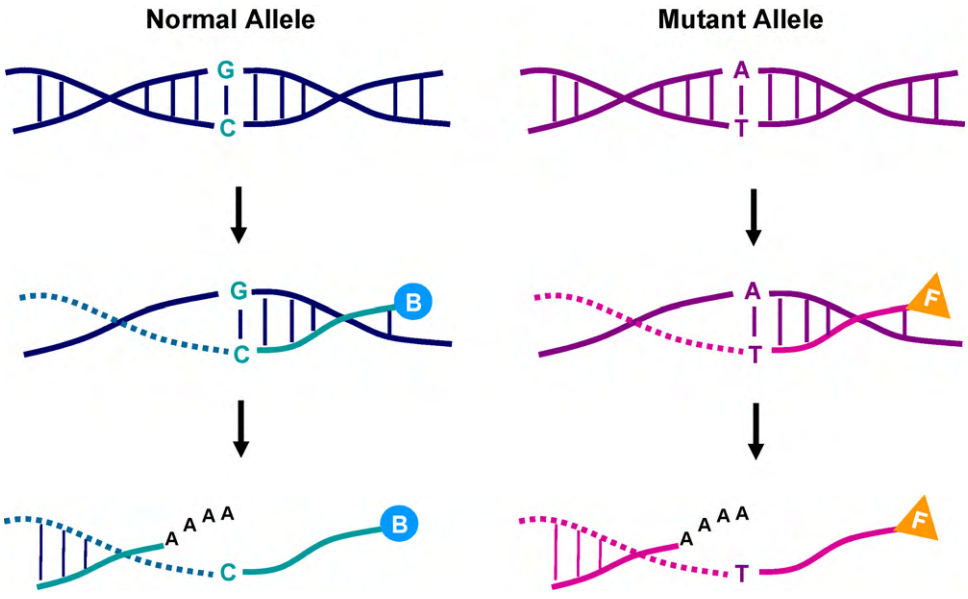

Fig. 1. Principle of allele discrimination by primer extension (PEXT) reaction on each locus. The products of a triplex PCR are heat-denatured and hybridized with allele-specific primers. The primers are 5′ labelled with biotin (normal primer) and fluorescein (mutant primer). A primer is extended only if the corresponding allele is present. The dotted lines represent the extended primer. The PEXT reaction mixture contains an oligonucleotide probe that is complementary to the extension segment of both primers and carries a poly(dA) tail at the 3′-end. B = Biotin; F = Fluorescein.

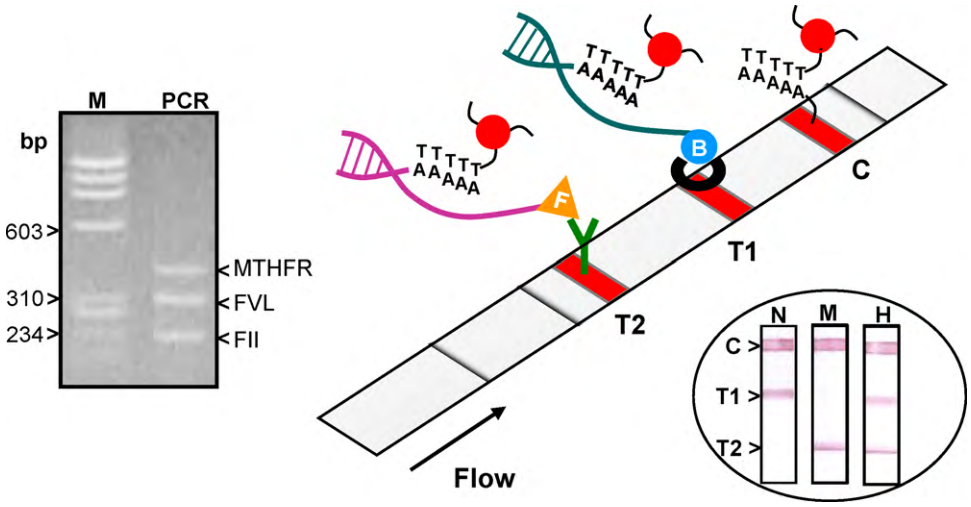

Fig. 2. Configuration of the dipstick-type DNA biosensor. Zones T1, T2 and C contain immobilized streptavidin, anti-fluorescein antibody and oligo(dA) strands, respectively. Oligo(dT)-conjugated gold nanoparticles are used as reporters for the visual detection of the genotyping reaction products by hybridization. Biosensor assay results for a normal (N), a homozygote mutant (M) and a heterozygote (H) for the MTHFR mutation are also presented. Moreover, an electropherogram (gel image) of the products of triplex PCR is shown. Lane 'M' presents the separation of DNA molecular weight markers. In the 'PCR' lane, the amplification products of the three genes (MTHFR, FVL and FII) are shown.

that carry poly(dT)-strands are deposited in dry form on the conjugate pad of the strip. The PEXT reaction product is applied to the conjugate pad, next to the nanoparticles, and then the bottom part of the strip is immersed into the hybridization buffer. Because of the capillary action, the buffer migrates upward allowing poly(dA)/(dT) hybridization and linking the PEXT product to the nanoparticles. The hybrids migrate along the strip to the test zones. The fluorescein- and/or biotin-labelled extension products are captured either by streptavidin or anti-FITC antibody immobilized on the T1 and T2 zones, respectively, and detected by the appearance of a red line due to the accumulation of gold nanoparticles. Both the anti-FITC antibody and streptavidin solutions contain sucrose that (a) helps maintaining the activity of the proteins during drying and heating and (b) facilitates the rehydration of the proteins during the dipstick test. The excess nanoparticles are captured by the immobilized poly(dA) strands at the control zone, thus forming a red band that indicates the proper performance of the strip. The presence of the normal allele is denoted by a red line at T1 whereas the presence of the mutant allele is signified by a red line at T2. The appearance of red lines in both T1 and T2 zones denotes a heterozygote for the mutation.

It should be emphasized that this assay configuration is different from the previously reported dipstick-type genotyping assays (Litos et al., 2007, 2009). In both previous reports the extension product is detected through the incorporation of biotin or hapten moieties (by means of modified dNTPs). On the contrary, in the present work the PEXT product is detected through hybridization with a specific oligonucleotide probe with no need for incorporation of modified dNTPs. This configuration enhances the specificity of the assay because it allows confirmation of the extended sequence.

3.2. Optimization studies

The dual-allele biosensor assay was optimized using genomic DNA samples, from a normal individual and a homozygote mutant for the MTHFR mutation.

Optimization studies were focused on the triplex PCR and PEXT reaction conditions in order to achieve the highest yield of amplification products and the best allele discrimination with the same thermal cycling parameters for the three PEXT reactions.

3.2.1. Triplex PCR

We found that at high concentration of PCR primers (>0.3 μM) the yield of the PEXT products decreases. This was attributed to the fact that during PEXT reactions the PCR primers are also extended and compete with the PEXT primer for the dNTPs and the enzyme. Also at high concentrations of PCR primers the formation of primer dimers is favored, which results in a lower yield of amplification product. The maximum yield of the PEXT products was observed with 0.2 μM of each primer.

The effect of Mg^{2+} concentration on the yield of the multiplex PCR reaction was studied in the range 1–3 mM. The maximum yield for all three products was observed at 2.5 mM Mg^{2+}. The effect of the annealing temperature for PCR was studied in the range of 53–66 °C and 55 °C was chosen as the optimum because the yield of the 319 bp amplicon corresponding to Factor V gene decreased at higher temperatures.

3.2.2. PEXT reaction

PEXT reaction was optimized with respect to several parameters such as annealing temperature, Mg^{2+} concentration, reaction primers and probe concentration. The annealing temperature was studied in the range 53–63 °C and the results are presented in Fig. 3. The variation of annealing temperature in the above range does not seem to influence either the nonspecific extension and/or the yield of the PEXT reaction. The effect of Mg^{2+} concentration was studied in the range of 0.8–2 mM using a series of PEXT reactions (for each mutation) from the same sample containing a constant amount of amplified target DNA (0.1 pmol) and ten times excess (1 pmol) of mutant and normal primer. In Fig. 3, the results obtained for each mutation at different Mg^{2+} concentrations are presented. It is observed that the changes in Mg^{2+} concentration do not affect the color intensity or the allele discrimination ability of the sensor (absence of nonspecific products in the entire range of Mg^{2+} concentration).

The next parameter that affects the kinetics as well as the specificity of the PEXT reaction is the primer concentration. We performed a series of PEXT reactions containing 0.1 pmol of target DNA and various concentrations of primers in the range of 0.05–0.4 μM. The products were analyzed by the dual-allele biosensor and the results are presented in Fig. 3. We observe that the intensity of the test zones is influenced by the concentration of primers. By

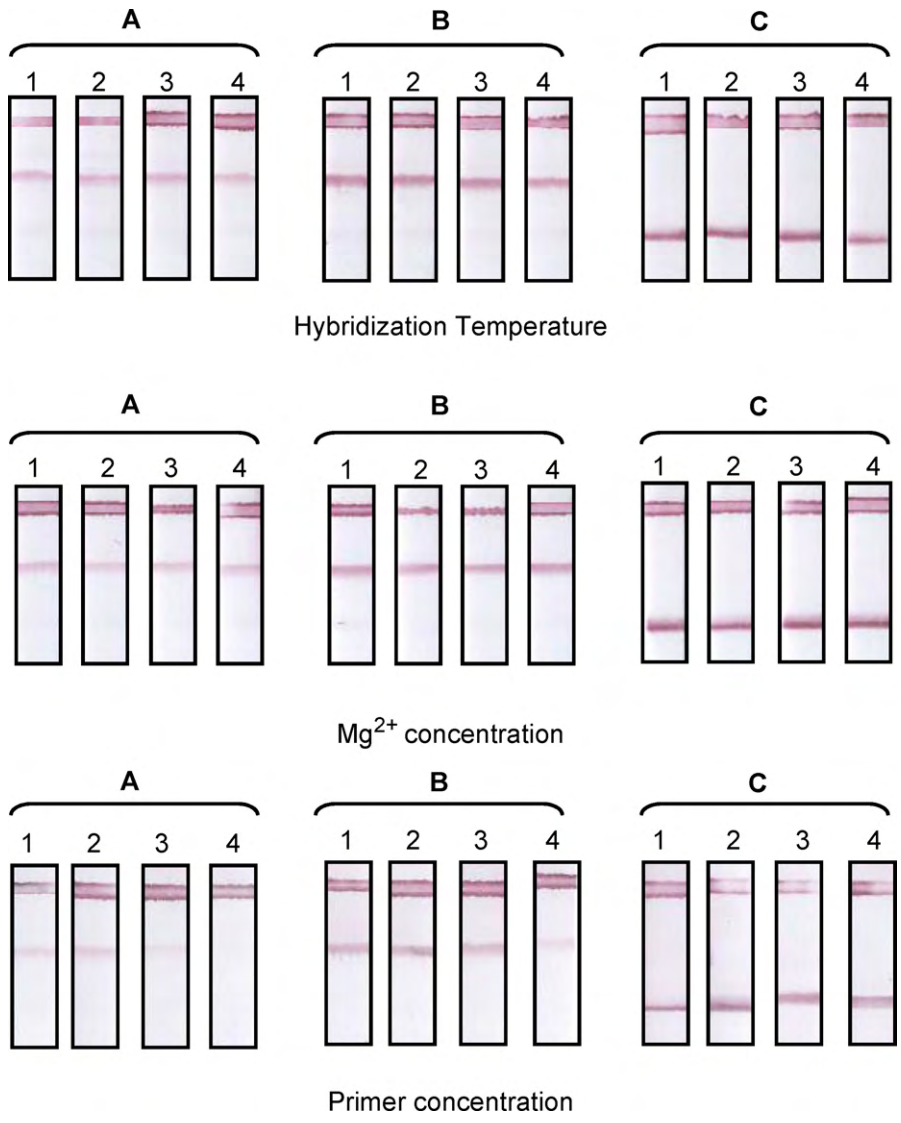

Fig. 3. Optimization of hybridization temperature, Mg^{2+} concentration and primer concentration for PEXT reaction. (A) FII gene, (B) FV Leiden (FVL), (C) MTHFR gene. Hybridization temperature: (1) 53 °C, (2) 55 °C, (3) 58 °C, (4) 63 °C. Mg^{2+} concentration: (1) 0.8 mM, (2) 1.0 mM, (3) 1.5 mM, (4) 2.0 mM. Allele-specific primer concentration: (1) 0.05 μM, (2) 0.1 μM, (3) 0.2 μM, (4) 0.4 μM.

increasing the primer concentration up to 0.1 μM, an increase of the zone intensity is observed due to increase of the reaction yield. At higher primer concentrations (>0.1 μM), the intensity of the test zones decreases due to the competition between free primers and the extended products for the limited binding sites on the test zones.

We also optimized the probe concentration in the PEXT reaction mixture in the range of 0.02–0.3 μM. The intensity of the test zone increases by increasing the probe concentration up to 0.13 μM due to the increase of hybridization yield. At higher probe concentrations the color intensity remains constant since all the extended products have been hybridized.

3.2.3. Dipstick assay

We optimized the amount of oligonucleotide-conjugated gold nanoparticles and the anti-FITC antibody, loaded on the conjugate pad and the first test zone, respectively. Genomic DNA from a normal individual was used for this study. The zone intensity increases with the amount of deposited nanoparticles. However, a faint nonspecific zone appears on the strip when more than 6 fmol nanoparticles were applied (Supplementary Fig. S1). Similarly, the zone intensity increases with the amount of anti-FITC antibody but a faint nonspecific zone is observed at amounts higher than >250 ng/4 mm strip when analyzing PEXT product for MTHFR mutation (Supplementary Fig. S2).

In order to eliminate the appearance of nonspecific signal on the antibody test zone, we studied the effect of the concentration of mutant primer (carrying the FITC label) in the PEXT mixture. The study was carried out with samples homozygous and heterozygous for this mutation using 250 ng anti-FITC antibody/4 mm strip. The concentration of the normal primer was kept at 0.1 μM, while the concentration of mutant primer varied in the range of 0.02–0.05 μM. It was observed (Supplementary Fig. S3), that at a concentration of 0.02 μM of the mutant primer the nonspecific signal is negligible, whereas both test zones for the heterozygous sample were of equal intensity.

For the small number of cycles of PEXT reactions (6 cycles), 0.25 U per reaction of DNA polymerase was found to be sufficient. At higher enzyme concentration, an increase of the nonspecific extension was observed.

The stability of the antibody that was immobilized on the test zone of the strip was studied by performing experiments of acceler-

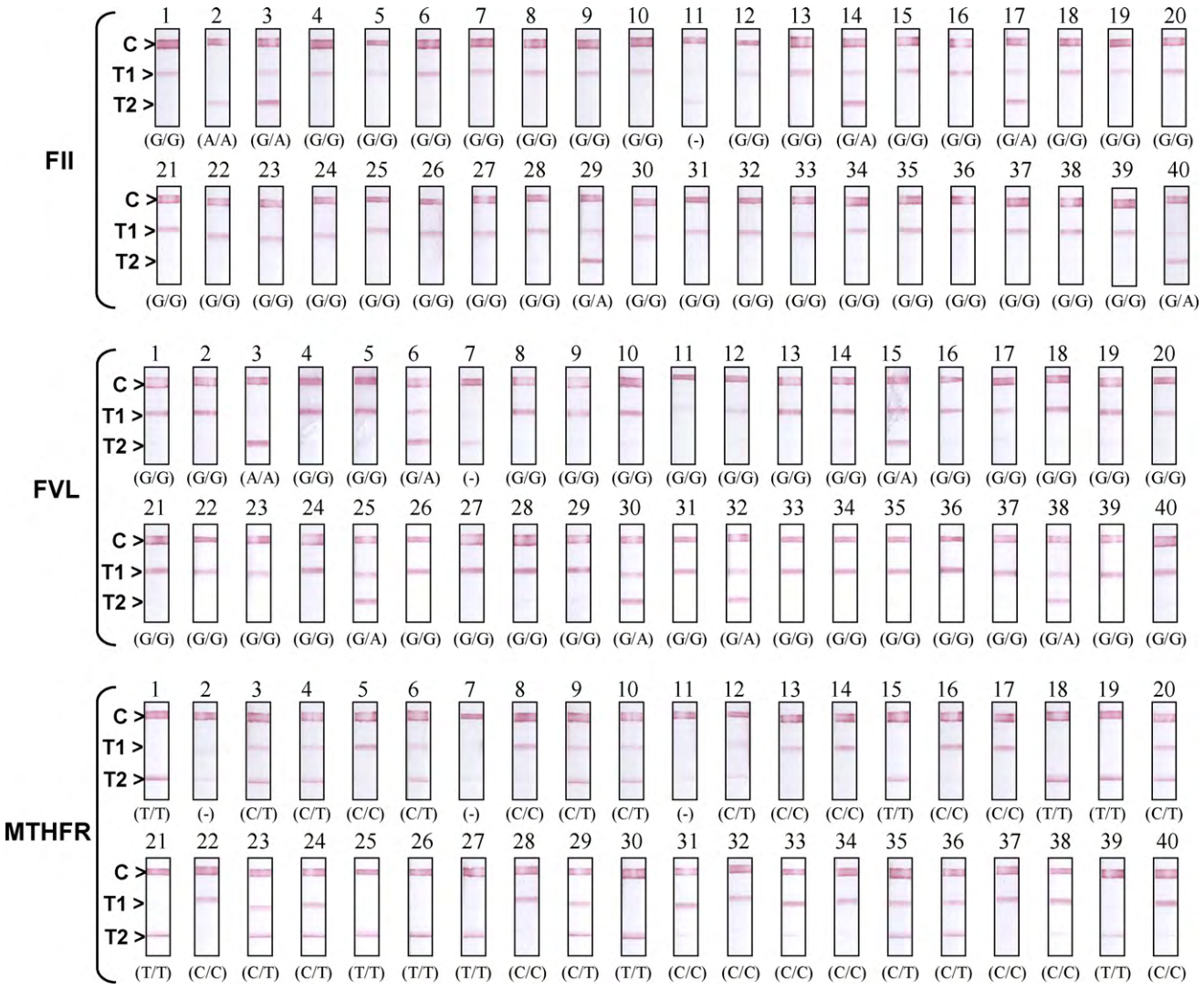

Fig. 4. Genotyping results of 40 clinical samples for the three mutations. Zones T1, T2 and C contain immobilized streptavidin, anti-fluorescein antibody and oligo(dA) strands, respectively and are shown by the corresponding arrows. Zones T1 and T2 denote the presence of the normal and mutant allele, respectively. The symbol (-) means that there was not enough genomic DNA for running the assay and obtaining clear genotyping results. The genotype shown below each strip was obtained from RFLP experiments. Sequencing was applied to those cases that RFLP results were not clear.

ated aging (Desfande, 1996). The strips were prepared as described in Section 2 and stored for 10 days at 36 °C. According to the accelerated aging procedure, these conditions correspond to 1 year storage at room temperature. We observed only a slight decrease in the signal after this storage period. The reproducibility of this type of dipstick assay for PEXT products is about 8% (coefficient of variation of the densitometric signals obtained from the test zones of the strip).

3.3. Application to clinical specimens

Genomic DNA specimens were obtained from the Hematological Laboratory-Molecular Biology Unit of Ioannina University Hospital. The proposed method was evaluated by genotyping 40 samples with a variety of genotypes for all three mutations (Fig. 4). Zones T1, T2 and C (shown by the corresponding arrows) contain immobilized streptavidin, anti-fluorescein antibody and oligo(dA) strands, respectively. Zones T1 and T2 denote the presence of the normal and mutant allele, respectively. A red line present only in zone T1 denotes a normal genotype (presence of the normal allele only). A red line present only in zone T2 denotes a mutant homozygote (presence of the mutant allele only). The appearance of red lines in both T1 and T2 zones denotes a heterozygote for the mutation. The genotyping results were in full concordance with those obtained by RFLP analysis and sequencing.

4. Conclusions

The proposed three-biosensor panel enables simple genotyping of 3 single-point mutations in the FV, FII and MTHFR genes, which constitute the most common genetic risk factors in thrombophilia. A triplex PCR was developed for simultaneous amplification of three fragments followed by a single PEXT reaction for each locus. Detection of the PEXT products was accomplished by means of a dual-allele dipstick-type DNA biosensor. No purification of the PCR products is required prior to the PEXT reaction. Also, the PEXT products are applied directly to the biosensor without purification. The biosensor is disposable and allows visual detection and confirmation of the genotyping products within minutes by hybridization without the need for specialized instruments or expensive reagents. The total time required for the genotyping after PCR is about 20 min. The assay does not require the use of biotinylated dNTPs during PEXT reaction. Instead, the PEXT primers are labelled and the products are detected through hybridization of the

extended segment with an oligonucleotide probe that is common for both alleles.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.06.020.

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