Four-Color Multiplex PCR Assay for the Simultaneous Detection of Four Allelic Variants in a Closed Tube Using a Single Thermal Cycler Program on the iCycler iQ™ Real-Time PCR Detection System

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Abstract

Homogeneous allele-specific assays for single nucleotide polymorphism (SNP) detection offer several advantages over the more traditional techniques based on the use of the polymerase chain reaction (PCR). For instance, labor is significantly reduced and the chances of generating carryover contamination are diminished because no post-PCR handling is required. The 5′ nuclease assay is one of the most widely used allele-specific homogeneous assays. The assay takes advantage of the 5′ → 3′ nuclease activity of *Taq* DNA polymerase to cleave fluorescently labeled allele-specific oligonucleotide (ASO) probes when they hybridize to PCR products during the PCR annealing phase.

We developed a real-time multiplex assay for the simultaneous detection of up to four allelic variants in one closed tube using a single thermocycling protocol and four probes each labeled with a different fluorophore. The assay combines the power of multiplex PCR with the specificity provided by ASO hybridization using the 5′ nuclease assay format. We applied the four-color assay for the simultaneous detection of the factor V Leiden (FVL) G1691A mutation (in the coagulation factor V gene) and the prothrombin (PT) G20210A mutation (in the coagulation factor II gene), the two most common known genetic risk factors for venous thrombosis in Caucasians. Human genomic DNA was prepared from whole blood using standard procedures. A 97 bp DNA sequence of the coagulation factor V gene was coamplified with a 111 base pair DNA sequence of the coagulation factor II (prothrombin) gene using four PCR primers. In addition, PCR reactions included four differentially labeled ASO probes for the specific detection of the different FVL/PT G20210A genotypes.

To evaluate the assay's performance characteristics, we performed a method comparison study. Results generated with the four-color multiplex assay were compared with those obtained with a reference method. We analyzed 52 DNA samples with known FVL/PT G20210A genotypes that were previously genotyped with the reference method. We found a 100% correlation between the results generated by both methodologies. We conclude that the four-color multiplex assay is specific and reproducible for the detection of the FVL/PT G20210A mutations, and it can be easily adapted for the detection of other SNPs. The four-color assay, which will be useful for both molecular diagnostic and research laboratories, offers numerous advantages over more traditional methods for the detection of the FVL and PT G20210A mutations. The advantages include speed and simplicity of the method, reduced labor, reduced risk of cross-contamination, and higher throughput.

Introduction

The development of new molecular tools and infrastructure for the identification of SNPs has facilitated the study of human variation. The uses of the study of SNPs are numerous; however, one of the most important SNP applications is in investigating the contributions of individual genes to diseases that have a complex, multigene basis. Variations in DNA sequence also play a role in how humans respond to disease; to environmental insults such as bacteria, viruses, toxins, and chemicals; and to drugs and other therapies. This makes SNPs of great value for biomedical research and for developing pharmaceutical products or medical diagnostics.

The introduction of homogeneous technologies that combine PCR with the use of fluorescent hybridization probes was a key turning point in the development of SNP detection technologies. Since their inception in research and molecular diagnostic laboratories, homogeneous methods for SNP detection have become the first choice among genotyping techniques because they offer several advantages over the more traditional genotyping techniques. For instance, labor is significantly reduced and the chances of generating carryover contamination are diminished since no post-PCR handling is required.

The 5′ nuclease assay (1) is one of the most widely used allele-specific homogeneous assays. The assay takes advantage of the 5′ → 3′ exonucleotic activity of *Taq* DNA polymerase to cleave fluorescently labeled allele-specific oligonucleotide (ASO) probes when they specifically hybridize to PCR products during the PCR annealing phase. In a typical 5′ nuclease assay for allelic discrimination, two dual-labeled fluorescent ASO probes — one specific for the wild-type (WT) allele and the other specific for the other allelic variant — are mixed in a PCR reaction with a pair of oligonucleotide primers. The probes are differentially labeled at the 5′ end with a reporter dye (6-FAM, HEX, Texas Red, Cy5, etc.) and with a quencher dye at the 3′ end (Black Hole Quencher 1, Black Hole Quencher 2, TAMRA, etc.). The close proximity of the reporter and quencher prevents fluorescent emission while the probes are not hybridized to a complementary DNA sequence. When the probes are hybridized to a complementary DNA sequence, cleavage occurs and the reporter and quencher dyes are separated. This results in an increased fluorescence of the reporter dyes as amplification proceeds.

We have recently described a four-color multiplex 5′ nuclease assay for the simultaneous detection in real time of four allelic variants in one closed tube using a single thermocycling protocol (2). In this poster, we report the development of a novel application of the multicolor assay for the simultaneous detection in real time of the factor V Leiden (FVL) and prothrombin (PT) G20210A mutations, the two most common known genetic risk factors for venous thrombosis in Caucasians. The assay, which can easily be adapted to the detection of other SNPs, will be useful for both molecular diagnostic and research laboratories.

Material and Methods

DNA Sample Preparation

Whole blood samples from 52 individuals with known FVL and PT G20210A genotypes were used for the preparation of human genomic DNA using standard procedures. Approximately 10 ng of each DNA solution (5 µl) was used for the multicolor multiplex assay.

PCR Primers and Probes

Oligonucleotide primers were designed with Oligo 6 software (Molecular Biology Insights, Inc., Cascade, CO). Synthetic oligonucleotide primers and probes were obtained from TriLink Biotechnologies, Inc. (San Diego, CA). The ASO probes were labeled with FAM/Black Hole Quencher 1 (FVL WT), TET/Black Hole Quencher 1 (FVL mutant), TAMRA/Black Hole Quencher 2 (PT G20210A WT), and Texas Red/Black Hole Quencher 2 (PT G20210A mutant).

PCR Annealing Temperature Optimization

The gradient feature of the iCycler iQ™ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) was used to optimize the annealing/probe hybridization temperature of the multiplex PCR reaction. Eight different annealing/hybridization temperatures ranging from 55 to 70°C were tested in one simple experiment by performing duplicate PCR reactions in real time. The multiplex PCR reaction ingredients and conditions used for the gradient experiment are described below.

Factor V Leiden and Prothrombin G20210A Genotyping by the Multicolor Multiplex 5′ Nuclease Assay

The multiplex PCR reactions were run in a 25 µl final volume containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 200 µM each dNTP, 0.4 µM each FVL and PT G20210A PCR primer, 0.4 µM each ASO probe, and 2.25 units of iTaq™ DNA polymerase (Bio-Rad, Hercules, CA). PCR reactions were performed in the iCycler iQ system using 96-well iCycler iQ PCR plates (Bio-Rad). Following *Taq* activation and DNA denaturation at 95°C for 3.5 min, the multiplex amplification-detection of both FVL and PT G20210A mutations was carried out for 50 cycles as follows: annealing and extension at 58°C for 45 sec and denaturation at 95°C for 10 sec. The fluorescent data generated by the cleavage of the dual-labeled ASO probes were collected during the PCR annealing step. Data analysis for allele discrimination was performed with the iCycler iQ software.

Genotyping by Restriction Fragment Length Polymorphism (RFLP)

Genotyping for the FVL and PT G20210A mutations by RFLP was performed as described previously (3) with some minor modifications. Briefly, the simultaneous amplification of two fragments of the coagulation factor V and factor II genes was performed in a multiplex PCR reaction. Subsequently, a 30 µl aliquot of each PCR reaction was mixed with 6 µl of 50 mM $MgCl_2$ and digested with *Hin*dIII at 37°C for 8 hr. The restricted PCR products were analyzed by electrophoresis in a 3% agarose gel followed by staining with ethidium bromide.

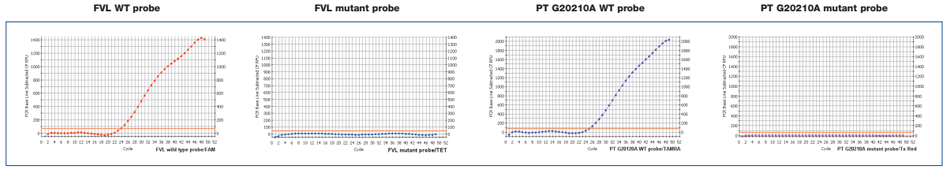

Fig. 1. WT genotype

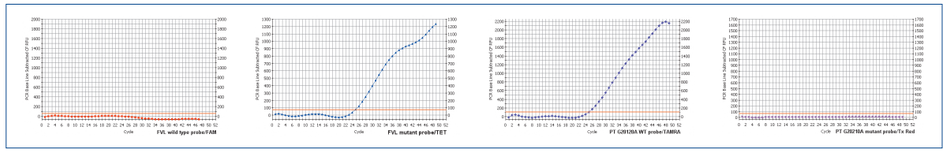

Fig. 2. Homozygous mutant FVL

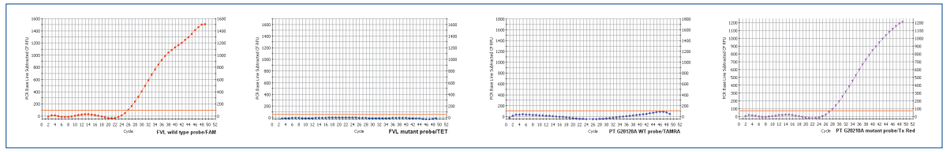

Fig. 3. Homozygous mutant PT G20210A

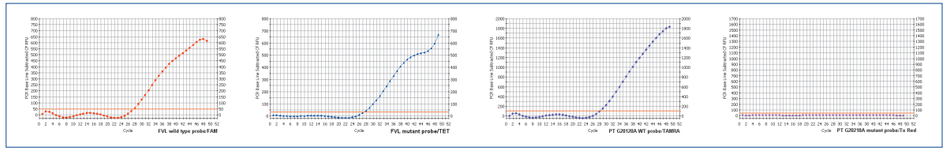

Fig. 4. FVL heterozygous genotype

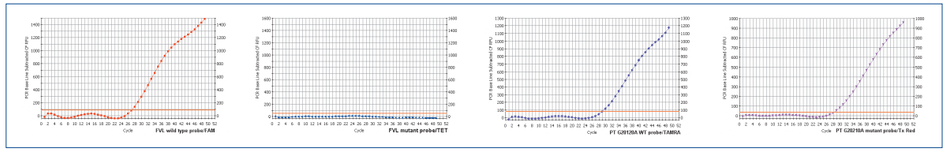

Fig. 5. PT G20210A heterozygous genotype

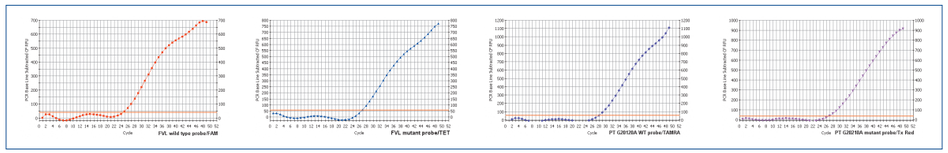

Fig. 6. FVL PT G20210A compound heterozygous genotype

Interpretations of the multicolor multiplex 5′ nuclease assay results for FVL and PT G20210A genotyping. Fluorescence (RFU) was measured every cycle and, after normalization, plotted against cycle number.

Results

Design of PCR Primers and ASO Probes

For primer design, the usual constraints and preventive measures were considered. For example, the formation of primer-dimers, hairpins, and self-complementary sequences was avoided, and the primers were designed to be compatible in a multiplex PCR reaction. Further, primers were chosen to synthesize PCR products with a length within the range of 90–150 base pairs. For the real-time detection of the FVL and PT G20210A mutations, ASO probes were designed to be complementary to sequences within the PCR products where the mutations occur. To maximize hybridization specificity, probes were designed such that the mismatch is positioned at a central location within the sequence of the oligonucleotide ASO probe. Probes were designed to be relatively short (17–19 nucleotides) with a melting temperature (T_m) of 55 ± 1.5°C. The T_m of the ASO probes was designed to be slightly higher than the T_m of the PCR primers.

Genotyping With the Multicolor 5′ Nuclease Assay

Six examples of outcomes obtained from the analysis of the FVL and PT G20210A mutations are shown in Figures 1–6. A WT individual (Figure 1) shows a fluorescent signal for both the FVL and PT G20210A WT probes but not for their mutant counterparts. A homozygous mutant individual for one of the two mutations (Figures 2 and 3) shows a fluorescent signal with the ASO probe complementary to the mutant sequence but not with its WT analog, and another fluorescent signal with the other WT probe but not with its mutant counterpart. Heterozygous individuals generate a fluorescent signal with both probes (Figures 4 and 5). Compound heterozygous individuals generate fluorescent signals with all four probes, the two ASO mutant probes and their WT counterparts (Figure 6).

Assay Performance

Assay performance characteristics were evaluated by performing a method comparison study. A published genotyping assay (3) that combines PCR with the use of restriction enzymes (PCR-RFLP) was selected as a reference method such that the genotyping results generated by the four-color assay could be compared. Results generated with the reference method showed that of the 52 DNA samples, 19 were heterozygous for PT G20210A, 19 were wild type, 3 were homozygous for FVL, 4 were homozygous for PT G20210A, 5 were heterozygous for FVL, and 2 were compound heterozygous. Figure 7 illustrates an example of the RFLP genotyping assay that shows the restriction patterns for some of the possible combinations of the WT, FVL homozygous mutant, and PT G20210A homozygous mutant genotypes.

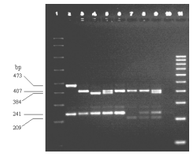

Fig. 7. A 3% agarose gel showing an example of different FVL/PT G20210A genotypes assigned with the RFLP reference method. Lane 1, 50–2,000 bp ladder (Bio-Rad); lane 2, undigested PCR products; lane 3, WT genotype; lane 4, homozygous mutant PT G20210A genotype; lane 5, heterozygous PT G20210A genotype; lane 6, WT genotype; lane 7, homozygous mutant FVL genotype; lane 8, heterozygous FVL genotype; lane 9, compound heterozygous FVL/PT G20210A genotype; lane 10, negative control; lane 11, 100–1,000 bp ladder (Bio-Rad).

After the completion of the PCR-RFLP study, the DNA samples were analyzed by the multiplex four-color assay. At the end of the PCR amplifications, the software for allelic discrimination automatically assigned the genotypes using an algorithm that considers the signal generated by replicates of a WT and homozygous mutant controls. The software is able to analyze either the final relative fluorescent units (RFU) or the threshold cycle values (C_T) and to plot the data as a diagram. Figure 8 depicts factor V genotype assignment by the software using the RFU mode. The plot shows four clearly defined clusters. The first cluster consists of samples with FAM signal values (x-axis) within the range 1,050–1,700 RFU, which represent individuals with the WT factor V genotype. The second cluster includes samples with TET signal values (y-axis) within the range 1,000–1,600 RFU, which represent individuals with the FVL homozygous mutant genotype. The third cluster (FVL heterozygous individuals) includes samples with FAM and TET signal values within the range 300–1,100 RFU. Finally, the last cluster represents samples with very low RFU values for both FAM and TET (no-template controls). Similarly, Figure 9 shows four well-defined clusters that clearly distinguish the different prothrombin G20210A genotypes. The genotyping results generated by the multiplex four-color assay and analyzed by the software were in agreement with the results of the RFLP analysis.

Assay Reproducibility

Six DNA samples from individuals with a known FVL/PT G20210A genotype (WT, FVL homozygous mutant, PT G20210A homozygous mutant, FVL heterozygous, PT G20210A heterozygous, and FVL/PT G20210A compound heterozygous) and a no-template control were used to examine the reproducibility of the four-color assay. The six DNA samples were tested five times each. For all cases, assays of the samples gave repeatedly consistent and unambiguous genotypes.

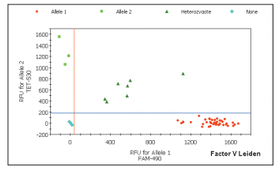

Fig. 8. FVL genotyping with the multicolor multiplex 5′ nuclease assay. Plot of fluorescence of FAM (FVL WT probe) vs. TET (FVL mutant probe). Normalized fluorescence values for both alleles were plotted along the x/y axes.

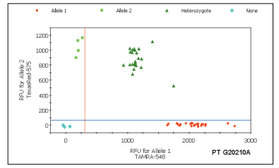

Fig. 9. PT G20210A genotyping with the multicolor multiplex 5′ nuclease assay. Plot of fluorescence of TAMRA (PT G20210A WT probe) vs. Texas Red (PT G20210A mutant probe). Normalized fluorescence values for both alleles were plotted along the x/y axes.

Conclusions

- We have shown the simultaneous detection of four allelic variants in real time using a single closed tube
- The multicolor genotypic 5′ nuclease assay for the detection of the FVL (G1691A) and PT (G20210A) mutations showed 100% specificity and 100% sensitivity as shown by the results of the comparison to the RFLP reference method
- The assay is reproducible
- The number of allelic variants to be detected in a 5′ nuclease assay depends on fluorophore choice and the design of the assay instrument. The four fluorophores selected for our multiplex multicolor genotypic assay have an excitation wavelength ranging from 488 nm (FAM) to 583 nm (Texas Red), and emission ranging from 515 nm (FAM) to 603 nm (Texas Red)

References

1. Holland PM, Abramson RD, Watson R, and Gelfand DH, Detection of specific polymerase chain reaction product by utilizing the 5′ → 3′ exonuclease activity of *Thermus aquaticus* DNA polymerase, Proc Natl Acad Sci USA 88, 7276–7280 (1991)

2. Ugozzoli LA, Chinn D, and Hamby K, Fluorescent multicolor multiplex homogeneous assay for the simultaneous analysis of the two most common hemochromatosis mutations, Anal Biochem 307, 47–53 (2002)

3. Dubreuil Lastrucci RM, Dawson DA, Bowden JH, and Marion Munster M, Development of a simple multiplex polymerase chain reaction for the simultaneous detection of factor V Leiden and prothrombin 20210A mutations, Mol Diagn 4, 247–250 (1999)

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