Detection of a point mutation in the prothrombin gene with the Agilent 2100 bioanalyzer

Application Note

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Abstract

This Application Note describes how the Agilent 2100 bioanalyzer can be used for the detection of a point mutation in the human prothrombin gene. The prothrombin protein plays a key role in blood clotting. A single nucleotide polymorphism (SNP), i.e. a point mutation in the prothrombin gene results in a common hereditary predisposition to venous thrombosis. We present a convenient and reliable mutation detection method by a PCR-based assay including a specific restriction digest (PCR-RFLP). Due to its high sensitivity and resolution, the applied microfluidic assay proves to be extremely suitable for detection of the obtained PCR products and restriction fragments. The developed method is suitable for the workflow at Laborigo molecular diagnostic laboratory.

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Introduction

The G20210A mutation in the untranslated part of the prothrombin gene results in an elevated serum prothrombin level and an increased risk for venous thrombosis. Individuals heterozygous for the prothrombin G20210A mutation have a two- to three-fold increased risk for venous thrombosis and elevated prothrombin serum level.[1,2,3,4] The prothrombin level of homozygous individuals is even higher, which probably leads to a higher risk of venous thrombosis. The risk for venous thrombosis is further increased in the presence of a second risk factor.[5,6] Some studies have shown that this mutation had an increased risk for arterial thrombosis[7] while other studies have not.[8] It is still under investigation whether an increased risk for arterial thrombosis exists only in circumstances where additional risk factors are present.[9]

DNA-based methods, such as the polymerase chain reaction (PCR)-based assay[2], are used to determine the presence of a specific mutation at nucleotide position 20210 in the prothrombin gene. This single nucleotide polymorphism allows the introduction of a new Hind III restriction site during PCR. If the Hind III restriction site is present this is an evidence of the prothrombin G20210A mutation. Heterozygous (two bands) and homozygous (one band) genotypes can be specifically identified. The two fragments potentially generated in this PCR-RFLP protocol are close in size. Therefore, we did a performance comparison between traditional agarose slab gel method and Lab-on-a-Chip technology using a microcapillary electrophoresis device.

Materials and methods

Sample preparation

Genomic DNA from blood samples of patients suffering from venous thrombosis and healthy controls was isolated using High Pure Template Preparation Kit (Roche). For each reaction a negative control containing distilled water was used. A heterozygous sample was used in the PCR as positive control.

PCR protocol

PCR reactions in a total of 15 µL were set up by mixing about 80 ng of extracted DNA, 7.5 µL of master mix (Promega) which contains $MgCl_2$ at a level of 1 mM and a primer master mix leading to a final concentration of 0.33 pmol/µL per each primer. Forward primer: 5'-TCTA-GAAACAGTTGCCTGGC-3' (93-787, nucleotides 19889-19908), reverse primer: 5'-ATAGC ACTGG GAGCA TTGAA*GC-3' (95-315, nucleotides 20233-20212, Laborigo, Hungary). The DNA was amplified under the following conditions: 95 °C for 5 minutes followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s and a final extension step of 72 °C for 5. The reverse primer's last nucleotides hybridize just adjacent with the potential point mutation in prothrombin DNA-strand. The nucleotide marked with an asterisk in the reverse primer is not present in the normal sequence. This allows the introduction of a new Hind III restriction site during PCR. The Hind III site (-A/AGCTT-) is completed in the amplified fragments in case of the less frequent allele (AAAG, mutation G20210A is present), yielding two fragments (322 bp and 23 bp in length) after respective enzymatic digestion. The more frequent allele (AGAG, wild type) lacks the restriction site and therefore generates only one non-digestible fragment of 345 bp.

Restriction digest

Restriction digest was performed in a total volume of 20 µL by adding a premixed digestion solution of 5 µL to the total volume of the PCR fragment containing 3 units Hind III and the recommended buffer R (Fermentas, Lithuania). Incubation at 37 °C was carried out for 4 hours.

Electrophoresis on agarose gel

Samples were run on 4 % precast long gel (BMA Reliant gel system) for 2 hours on 100 V, in the presence of ethidium bromide. After restriction digest wild type samples showed a 345 bp long normal allele, whereas heterozygous samples contained both, a normal and

mutant allele with 345 bp and 322 bp products respectively. Due to the little size difference 2 hours run is needed for sufficient separation.

Electrophoresis using the Agilent 2100 bioanalyzer

Double stranded DNA samples (PCR-products and restriction digests) were run on the Agilent 2100 bioanalyzer using the DNA 1000 LabChip kit in accordance with the manufacturer's instructions. Briefly, 9 µL of the gel dye mixture was added to the priming well. With the chip priming station provided this well was pressurized for one minute. After this the micro-channels are filled. Then 9 µL of the gel dye mixture were added to the other two remaining chip wells for gel labeled with "G" and to the ladder well. 1 µL of the provided ladder was added to the ladder well. Subsequently 5 µL of the marker solution were added to each of the twelve sample wells. 1 µL of each sample was added to their corresponding wells on chip. The chip was vortexed for one minute at the recommended setting of 2400 rpm. The chip was placed in the Agilent 2100 bioanalyzer and the double stranded DNA 1000 assay was started. Twelve samples were run within 30 minutes. Digital data were automatically analyzed and archived using the Agilent 2100 expert software.

Results and discussion

Our established PCR assay followed by RFLP analysis enables the detection of a SNP, situated at the 20210 nucleotide position of the prothrombin gene. The significance of the diagnostics relies on accurate interpretation of visual data. The sizing differences as well as the intensity of heterozygous bands are difficult to discern on the agarose gel.

After the PCR–RFLP steps, 2 hours running of the products on an agarose gel were needed to achieve relatively good resolution for the two closely sized products. Gel documentation (CCD camera, UV-transilumination stage) took an additional 10 minutes, and if quantitation was also required, it had to be analyzed for an additional 15 minutes. Since in our laboratory the traditional method was time-consuming and involved the potentially hazardous ethidium bromide, we searched for a more convenient alternative. We preferred to run our samples on the Agilent 2100 bioanalyzer, where separation at a good resolution and precise qualitative and quantitative analysis, as well as digital archiving, is directly available.

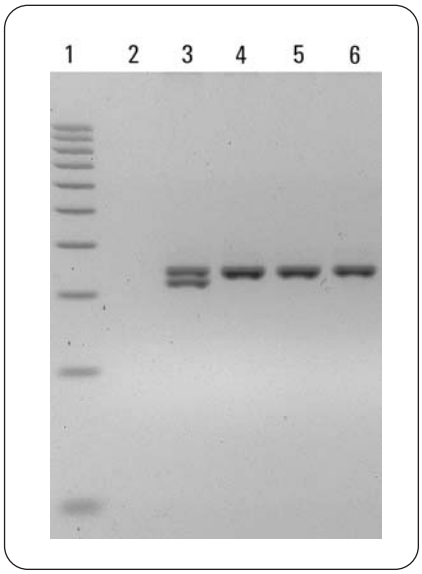

Figure 1
Traditional analysis of DNA fragments. Applied samples are DNA size marker (1), negativ control reaction (2), PCR-RFLP from heterozygous patient with fragments for wild type and G20210A point mutation with 345 and 322 bp products (3), PCR-RFLP from homozygous wild type patients (4, 5 and 6). Separation was done for 2 hours on a precast 4 % agarose gel with ethidium bromide staining during the run.

From figure 1, lane 3 it is obvious that the PCR products are only slightly different in size and therefore unambiguous evaluation is sometimes difficult to achieve, even if optimal separation time of 2 hours is applied. Lanes 4, 5 and 6 show the normal, non-mutant genotype of the prothrombin gene.

In applying the microfluidic technology of Agilent's equipment, however, we were able to easily detect point mutation and make reliable evaluation of the SNP situated in the relevant gene. Figure 2 shows the results of the separation on the LabChip device, i.e. ladder, negative control, a heterozygous control and individual samples with widespread wild type genotypes and a rare homozygous mutant sample (sample 10) from different patients. Here, an intuitive result flagging with customizable color coding for the presence of single or multiple fragments within a certain size range was used for automated evaluation. The sizing for all bands resulting from the normal prothrombin gene was highly reproducible: 345.9±1.1 bp. This value is in good agreement with the expected size of 345 bp. Quantitation of the product yield is also available by the software. The product concentration yielded by PCR were ranged from 2.5 to 3.5 ng/µL with a mean of 3.1±0.4 ng/µL. Availability of these values may help to meet requirements for quality control purposes, e.g. determining a minimum amount of product that must be present. The resolution achieved by the

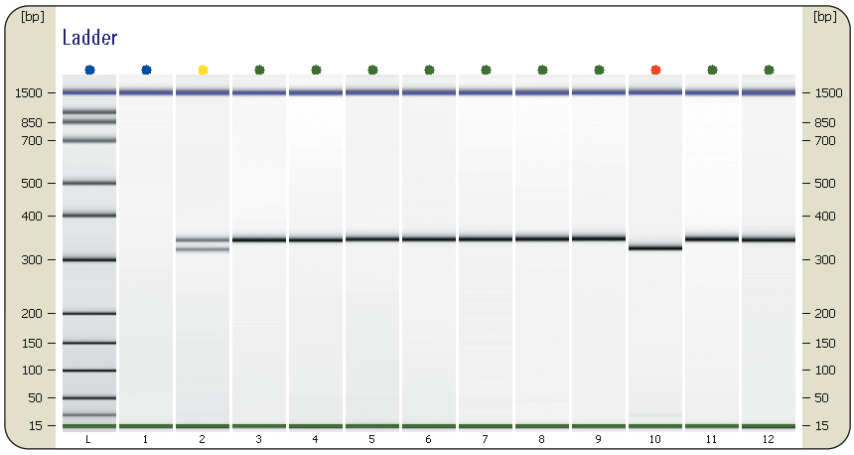

Figure 2
G20210A-screening, PCR-RFLP results from individual patients.
The gel like image from the Agilent 2100 expert software show ladder (L), negative control (1), heterozygous control (2) and patient samples (3-12) with a positive screening result for sample 10. Automated result flagging was applied and leads to evident overview: green dot (3-9, 11, 12): homozygous wild type = normal thrombosis risk; red dot (10): homozygous with G20210A point mutation = high thrombosis risk factor; yellow dot (2): heterozygous, wild type and G20210A point mutation = elevated thrombosis risk factor; blue dot (L, 1) none of the expected fragments (345/322 bp) are present. This last flagging rule detects the ladder and negative control.

DNA1000 assay allows precise baseline resolved separation of the expected fragments of 345 and 322 bp (figure 3). The visualization of the 23 bp fragment cleaved off during the PCR-RFLP protocol is also possible with the Agilent 2100 bioanalyzer. This is a further confirmation of a successful reaction which is not possible at all with agarose gel electrophoresis.

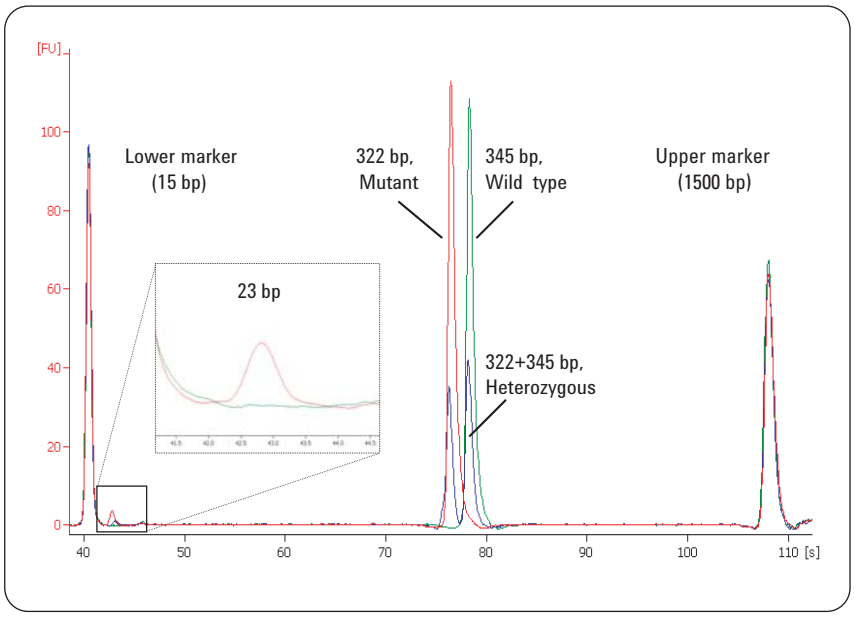

Figure 3
Resolution and detection capabilities.
Electropherograms of G20210A PCR-RFLP analysis of selected samples: wild type (green), heterozygous (blue) and homozygous, with G20210A point mutation (red). Expected fragments of 345/322 bp are baseline resolved. Sizing capabilities of the DNA1000 assay allow visualization of the 23 bp fragment (insert).

Conclusions

For the identification of the G20210A point mutation in the prothrombin gene, two DNA fragments of 345 bp and 322 bp must be completely separated and correctly sized. This application type is extremely important in making a genetic diagnosis for patients with venous thrombosis. In this study, we used microfluidic on-chip electrophoresis on the Agilent 2100 bioanalyzer for fragment separation and compared the results to traditional slab-gel electrophoresis. The data obtained from these studies show in detail that the Agilent 2100 bioanalyzer can distinguish between mutated and normal DNA samples. Furthermore, the results are superior in terms of sizing accuracy, quantitation capability, reproducibility and resolution compared to slab gel analysis. Data is generated in a digital format, so there is no further need for any other archiving process or instrument which streamlines the complete workflow. In addition, our results prove that application of the Agilent 2100 bioanalyzer in conjunction with a PCR-RFLP approach is a rapid and accurate diagnostic method, even if closely located DNA bands have to be distinguished. These findings suggest that the Agilent 2100 bioanalyzer could also be adopted and easily used for further SNP detection assays.

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