α1-Antitrypsin Mutation Detection on the LightCycler Using Human Genomic DNA from Blood Research Samples Isolated on the MagNA Pure LC

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The performance of the MagNA Pure LC for the isolation of genomic DNA from human EDTA blood for mutation analysis on the LightCycler has now been evaluated. Sample volumes of up to 200 µl EDTA blood gave reproducible high yields of pure DNA. The isolated DNA was stable at –20 °C without signs of degradation or weakness in the performance in the LightCycler, even after 9 months, the time since the MagNA Pure LC has been available. The yield of DNA was higher than from kits with manual extraction. We have developed protocols on the LightCycler for mutation analysis using DNA isolated on the MagNA Pure LC. These applications include a variety of genes, like α1-antitrypsin deficiency, methylenetetrahydrofolate reductase, apoB3500 and apoE, prothrombin and other genes of scientific interest. Here we report a LightCycler hybridization probe for discrimination of two mutations in the Z-allele site of the α1-antitrypsin gene (PI).

Introduction

In large projects, where hundreds and thousands of samples are being dealt with, robotics for sample handling without the risk of contamination and homogeneous and fast systems for PCR amplification are inevitable. For the rapid screening of large numbers of samples for specific mutations or polymorphisms, automated PCR and nucleic acid extraction platforms, like the LightCycler and MagNA Pure LC, are being used. Analysis of sequence variations (e.g., SNPs) in the context of the human genome project is an emerging field that promises to add to the understanding of biological processes that lead to favorable and unfavorable phenotypes. Applications for the screening of a large number of samples are numerous in the field of pharmacogenetics, and research studies on e.g., metabolic disorders, cancer, inherited diseases or infectious diseases.

The LightCycler technology is being widely used for mutation/polymorphism analysis because of its ease of use, speed and contamination-free, homogeneous platform [1]. Protocols have been established on the LightCycler to analyze mutations or polymorphisms in genes relevant to cardiovascular abnormalities, thrombosis, liver cirrhosis, pulmonary emphysema and susceptibility to metabolic disorders [2-6].

Protease inhibitor 1 (α1-antitrypsin, AT) is the main serum inhibitor of proteolytic enzymes. In AT-deficiency, enzymes like neutrophil elastase can damage the lung tissues leading to pulmonary emphysema [7]. The three most important variants in the PI-gene (protease inhibitor 1 gene) are type M (90 % of population), type S (PI*S) and type Z (PI*Z). Homozygotes of type Z have a considerable reduction in the serum α1-antitrypsin concentration and may develop pulmonary emphysema or hepatic cirrhosis. SZ-heterozygotes are less severely affected [8-9]. DNA isolated from human blood has been used on the MagNA Pure LC for the detection of mutations underlying the PI*Z-allele (Glu342Lys, GAG to AAG) and the PI*S-allele (Glu264Val, GAA to GTA) [5]. We were recently able to identify a second mutation covered by the allele Z detection probe (Asp341Glu, GAC to GAG), which was only one nucleotide apart from the Z-allele mutation.

The use of the MagNA Pure LC as a fully automated DNA extraction system for the isolation of human genomic DNA from blood for mutation analysis in the protease inhibitor gene (PI) and the factor V gene on the LightCycler is described.

Materials and Methods

DNA preparation from EDTA blood
Human genomic DNA was routinely isolated on the MagNA Pure LC from 200 µl EDTA blood using the MagNA Pure LC DNA Isolation Kit I either with the fast protocol or with the newly developed high performance

protocol following the instructions of the corresponding package inserts.

For the evaluation of both isolation protocols, sample volumes of 20, 50, 100 and 200 µl EDTA blood from the same individual with an average leukocyte count (5.5×10^6/ml) were used.

Mutation analysis for factor V Leiden and PI*Z-allele on the LightCycler

The quality and quantity of the isolated DNAs from the MagNa Pure LC was checked by agarose gel electrophoresis and by performance in a LightCycler application (factor V Leiden). The DNA isolates were either fresh, or kept at –20 °C for up to 9 months. 2 µl of the 100 µl DNA eluate were used in a standard 20 µl LightCycler PCR. For factor V Leiden mutation detection, the corresponding kit from Roche Molecular Biochemicals was used. The PI*Z-allele analysis was done as previously published [5]. The PCR primers were 5'-TCCACGTGAGCCTTGCTCGAGGC-CTG-3' (forward) and 5'-TTGGGTGGGATTCACCACTTTTC-3' (reverse), generating a 253-bp PCR product. The hybridization probes were CTCCAGGCCGTGCATAAGGCTGT-F (anchor) and RED640-GACCATCGACGGAAAGGG-p (detection). The LightCycler-DNA Master Hybridization Probes Mix was used.

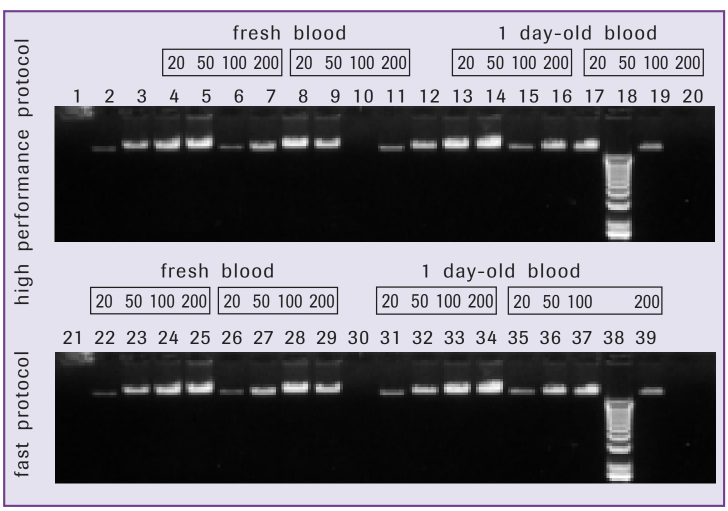

Figure 1: Agarose gel electrophoresis (0.7 %) of DNAs isolated from 20, 50, 100 and 200 µl EDTA blood on the MagNA Pure LC. For MagNA Pure LC-isolated samples, 10 µl of the 100 µl DNA eluate were loaded on the gel (equals one tenth of the amount of blood sample used). Lane 1 and 38 contain a molecular weight standard (1 kb ladder), lane 2 contains DNA isolated by manual extraction with reagents from another supplier and corresponds to 20 µl EDTA blood in total. Lanes 3, 12, 21 and 30 correspond to preparations where elution buffer instead of human blood was used. Lanes 4-11 (fresh blood) and 13-20 (1 day-old blood) reflect duplicate DNA isolations of 20, 50, 100 and 200 µl blood DNA with the high performance protocol, while lanes 22-29 and 31-39 reflect the corresponding samples with the fast protocol

Results and Discussion

Reproducibility and quality of DNA preparations

In order to check the reproducibility of DNA preparations by the MagNA Pure LC Instrument, freshly drawn and 24 hour-old human EDTA blood, maintained at 4 °C, with an average leukocyte cell count (5.5×10^6/ml) was employed. Sample volumes of 20, 50, 100 and 200 µl were used in duplicate for DNA extraction with two different extraction protocols, namely the fast protocol and the high performance protocol, both running with the MagNA Pure LC DNA Isolation Kit I. One tenth of the DNA eluate was analyzed on an agarose gel (Figure 1).

As demonstrated in Figure 1, all preparations yield excellent DNA that is free of degradation. According to ethidium bromide staining the DNA yield with the high performance protocol is by a factor of 2 higher compared to the fast protocol (lanes 3-20 versus lanes 21-39). The DNA isolation and efficiency on the MagNA Pure LC is not affected negatively when one day-old blood is used (lanes 3-11 versus 12-20) and the DNA yield is dependent on the sample volume used. In addition, from a 200 µl sample preparation (e.g. lane 7), as much DNA is isolated as with comparable manual extraction protocols (see lane 2), but the DNA shows no signs of degradation. In these two lanes, the DNA corresponds to the same input sample volume of 20 µl blood.

The various DNA preparations (2 µl each) were used in a factor V Leiden mutation analysis on the LightCycler. Representative results from DNAs isolated with the high performance protocol from the one day-old blood are shown in Figure 2. The corresponding DNAs were used in triplicate together with a heterozygous control and 3 standard DNAs of known concentration. The crossing points were in agreement with the DNA yield. Melting curve analysis showed good correlation with the melting peaks.

Stability of DNA samples and reanalysis

In certain cases, it may be necessary to reanalyze the DNA isolates that have been stored for long time, therefore stability of the DNA is important. Analysis of fresh DNA isolates and isolates that have been kept frozen at –20 °C for 6 and 9 months was carried out on an agarose gel and on the LightCycler (factor V Leiden mutation). As shown in Figure 3, the DNA is not degraded and is suitable for factor V Leiden mutation analysis. The differences in DNA yield are due to steadily improved extraction protocols and are not a result of degradation, which should result in a smear on the agarose gel.

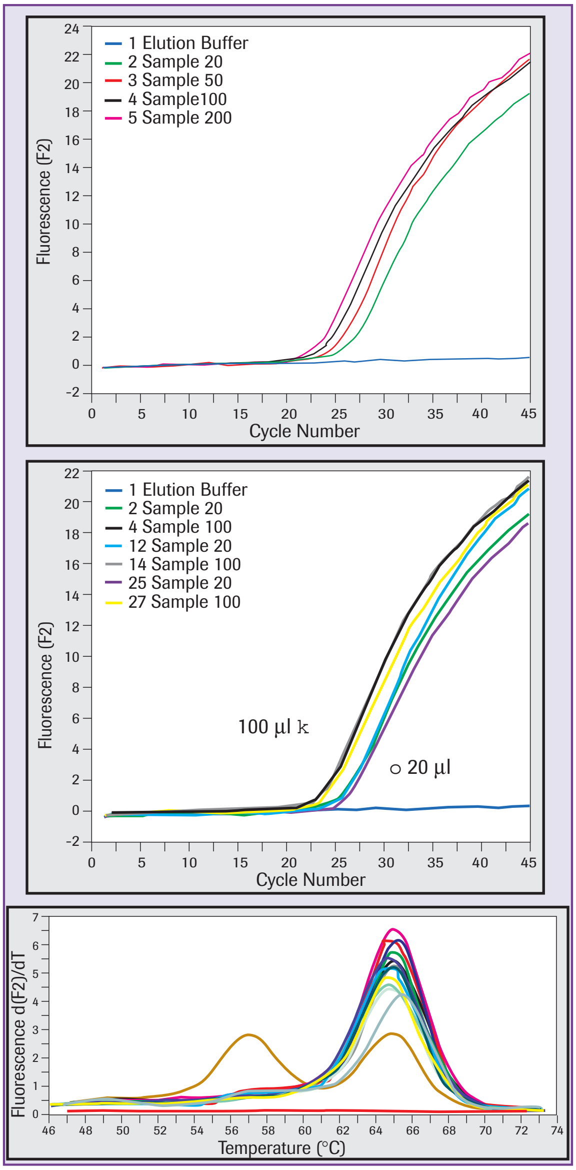

Figure 2: Factor V Leiden mutation analysis of DNAs isolated from 20, 50, 100 and 200 µl EDTA blood on the MagNA Pure LC. DNAs were isolated from one day-old blood using the high performance protocol (see Figure 1). Top panel displays representative fluorescence curves from DNA samples isolated from 20, 50, 100 and 200 µl blood. Middle panel displays the fluorescence curves of triplicate LightCycler analyses of DNA samples isolated from 20 µl and 100 µl blood. Bottom panel displays the melting curves of all samples

α1-Antitrypsin mutation analysis on the LightCycler

In the course of the analysis of the PI*Z-allele on the LightCycler in DNA samples from the MagNA Pure LC, we were able to identify a so far undescribed mutation/polymorphism that locates only one nucleotide upstream of the mutation leading to the Z-allele (GAG to AAG, Glu342Lys). Thus, using one hybridization (detection) probe, it was possible to discriminate between the two mutations. Figure 4 shows the fluorescence curves and the melting curves of a wild type individual, a heterozygous individual for the Z-allele and the "aberrant" melting peaks of an individual with a new PI-allele (Asp341Glu). While the G-T mismatch in the Z-allele leads to a shift of 7 °C in the melting peaks, the newly identified C-C mismatch in the PCR product of the later individual leads to a more dramatic shift of 12 °C and enables the discrimination of the different mutations. Sequencing of the 253-bp PCR product revealed a C to G transversion in the third position of codon 341 in the PI-gene (GAC to GAG, Asp341Glu) (Figure 4). As can be seen also in the fluorescence curves, the destabilizing effect of the C-C mismatch in the new PI-allele reduces the fluorescence intensity (data not shown).

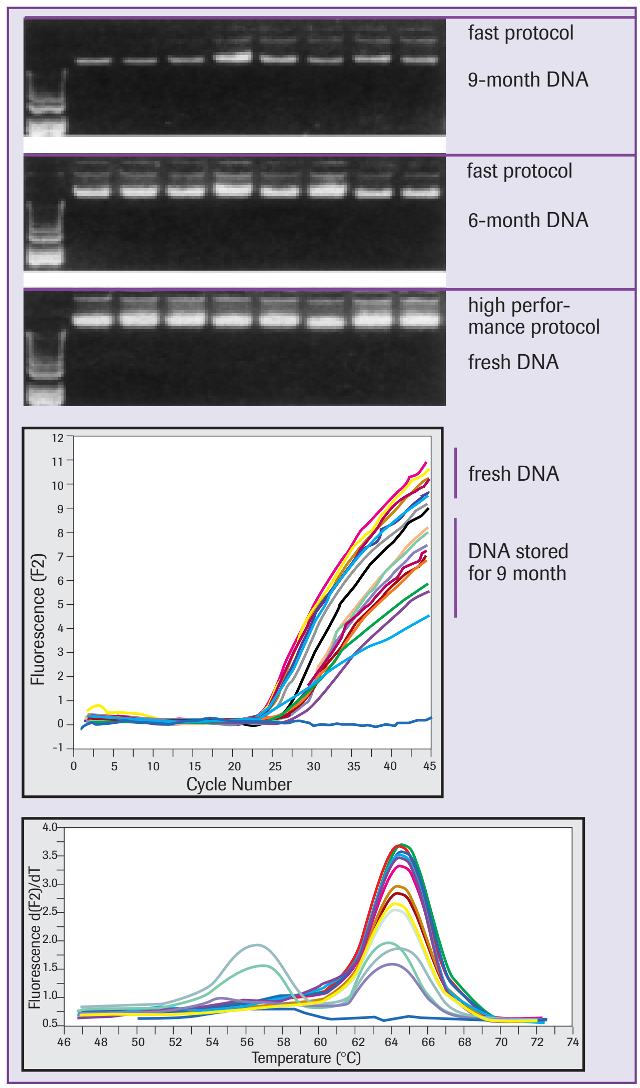

Figure 3: Agarose gel electrophoresis and factor V Leiden mutation analysis of frozen DNA samples isolated on the MagNA Pure LC from 200 µl EDTA blood. All DNAs were isolated from different individuals. Differences in DNA quantity are due to steadily improved protocols

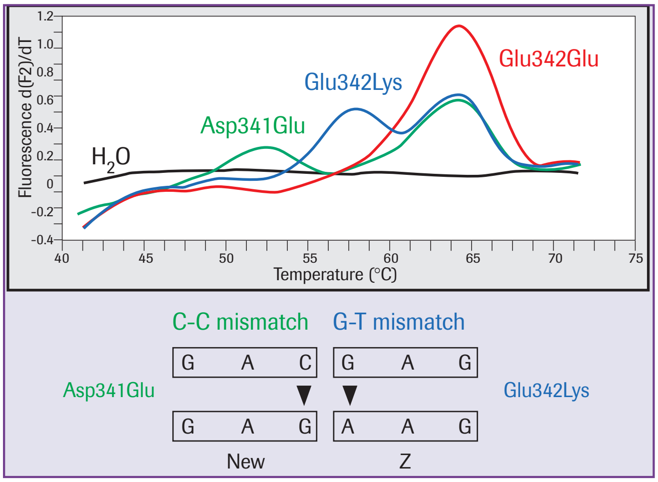

Figure 4: α1-Antitrypsin (PI) gene mutation analysis and DNA sequence surrounding the Z-allele mutation site in three individuals. The hybridization probe was derived from the wild type sequence of the PI*Z-allele (Glu342). Results from a homozygous wild type (Glu342Glu), a heterozygous for the Z-allele (Glu342Lys) and a heterozygous for a new PI-allele (Asp341Glu) are displayed

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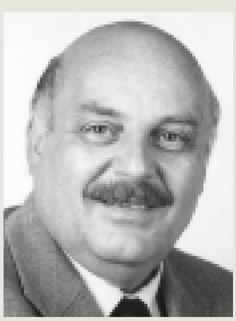

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Conclusions

The combination of two high-speed systems like the MagNA Pure LC and the LightCycler, for efficient DNA preparation and mutation analysis, has contributed considerably to the ease and reproducibility of analysis in laboratories dealing with large numbers of samples. It has been demonstrated here that the DNA preparations from the MagNA Pure LC are of high purity and yield and suitable for long-term storage. This is particularly important due to the current scientific focus on new genes and genomes, as a result of which more new mutation/polymorphism analyses will have to be performed from DNA samples in the future. Any competitive method should allow allele determinations rapidly and economically. The MagNA Pure LC and the LightCycler are perfect tools for reliable and accurate genotyping in research applications.

References

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Product	Pack Size	Cat. No.
LightCycler-DNA Master Hybridization Probes	1 kit (96 reactions)	2 015 102
	1 kit (480 reactions)	2 158 825
LightCycler-Factor V Leiden Mutation Detection Kit	1 kit (32 reactions)	2 212 161
LightCycler Instrument	1 instrument	2 011 468
MagNA Pure LC Instrument	1 instrument plus accessories	2 236 931
MagNA Pure LC DNA Isolation Kit I	1 kit (192 reactions)	3 003 990