

Analysis of the Mutation in the Human DPYP Gene from Human Blood Using the InviMag® Blood Mini Kit with the Thermo Scientific KingFisher® mL Instrument

By Dr. H. Hannig (Central Institution for Molecular Diagnostic, Clinic Braunschweig GmbH), Sonja Farhangi (Invitek GmbH, Robert-Rössle Str. 10, DE-13125 Berlin, Germany), and Laura Kanto, M.Sc. (Tech.) (Thermo Fisher Scientific, Ratastie 2, FI-01621 Vantaa, Finland)



Abstract

This application note describes the isolation of DNA from human whole blood samples and detection of the dihydropyrimidine dehydrogenase gene (DPYD) exon 14 skipping mutation using the InviMag Blood Mini Kit/ KFMl (Invitek) and the Thermo Scientific KingFisher mL magnetic particle processor (Thermo Fisher Scientific) combined with a PCR analysis. In this experiment up to 15 samples were processed on the KingFisher mL in an automated operation. The results show that the extracted genomic DNA can be used for mutation screening of the DPYD gene prior to 5-fluorouracil (5-FU) treatment in cancer patients.

Introduction

In accordance with the discovery of pharmacogenetic polymorphisms and a better understanding of interindividual variability in drug metabolism, new molecular techniques allow prediction of individual metabolic capacity. Particularly with regard to therapeutic purposes, these methods

require extremely high efficiency and sensitivity for isolation and detection of nucleic acids in a large number of samples. The human dihydropyrimidine dehydrogenase gene (DPYP) encodes the dihydropyrimidine dehydrogenase (DPD, E.C. 1.3.1.2) which is the initial and rate-limiting enzyme in the three-step metabolic pathway leading to the catabolism of the pyrimidine bases uracil and thymine. This is the only metabolic pathway in the biosynthesis of β -alanine in mammals [1]. DPD is also the key enzyme that degrades the structurally related pyrimidine anti-metabolite 5-fluorouracil (5-FU), a common anti-cancer drug used in the treatment of colon, breast, head, neck, and ovary tumors [2]. Less than 10% of administered 5-FU undergoes renal excretion, whereas > 80% is cleared by catabolic degradation by DPD to fluorinated β -alanine [3]. The deficiency in DPD enzyme activity is correlated with a delay in the clearance of 5-FU from the plasma. Accordingly, toxic side effects (for example, diarrhea, stomatitis, mucositis, myelosuppression, and neurotoxicity) of 5-FU have been linked to low levels of DPD enzyme activity in human blood cells [3].

The most common mutation associated with DPD deficiency is a point mutation within the GT 5'-splicing consensus sequence of exon 14 (G to A), IVS14 + 1 G > A, DPYD*2A [4]. This mutation results in the skipping of the

entire exon 14 and causes a deletion of 55 amino acid residues in the primary sequence of the DPD protein, resulting in a lack of functional DPD expression [3]. Even heterozygosity for this mutation can result in a large decrease in DPD activity and an increase in toxicity to 5-FU [5].

The pharmacogenetic syndrome of DPD deficiency has been detected in 3 – 5% of the population [6]. Therefore, screening for the DPYD*2A mutation is routinely done prior to 5-FU treatment in cancer patients. 5-FU administration can often be safely continued with an individual dose adjustment.

For these routine screening purposes, semiautomatic purification of blood samples with a KingFisher mL, from Thermo Fisher Scientific, in combination with the InviMag Blood DNA Mini Kit/ KFMl, from Invitek, were implemented in 2004. Compared to using fully automatic machines, this system provides a cost-effective step towards the standardization of labor-intensive nucleic acid purification.

Material and Methods

DNA purification from human whole blood

Genomic DNA is purified from human whole blood samples in 25 minutes using the commercial InviMag Blood DNA Mini Kit/ KFMl. The samples are obtained from cancer patients who should be treated with 5-FU. The quality of the incoming samples differs

widely (differences in health of the donor, age, sample storage and transportation).

After mixing 150 µl EDTA blood with 200 µl Lysis Buffer A and 20 µl Proteinase K, the samples are incubated covered while continuously shaking at 900 rpm for 10 min at 56°C to support proteinase activity for lysis and protein digestion. During lysis an appropriate number of Thermo Scientific KingFisher mL tube strips needed for the samples (one tube strip per sample) are placed into the tube strip tray. The tube strips B to E are filled with buffers supplied with the kit according to Table 1. After lysis the samples are transferred into KingFisher mL tube strips (Tube A) and 400 µl of Binding Buffer B6 and 20 µl MAP Solution A are added and mixed with each lysate to adjust the binding conditions.

After filling the tube strips, the tray is placed into the instrument and the tip combs are inserted into the slots. The front lid is closed and the samples processed using the “InviMAG_Blood_KFmL” purification protocol. Under these conditions genomic DNA is bound to the magnetic particles quantitatively. During the process, the beads are transferred through Wash Buffer I and Wash Buffer II to remove all impurities, such as proteins, nucleases, and PCR inhibitors. After removal of ethanol from the beads, the genomic DNA is eluted in Elution Buffer

Table 1 : Pipetting instructions for the InviMag Blood DNA Mini Kit/ KFmL

| Tube | Content | Sample/ Reagent volume |
|------|-------------------------------|------------------------|
| A | Lysed sample | 370 µl |
| | MAP Solution A ¹ | 20 µl |
| | Binding Buffer B6 | 400 µl |
| B | Wash Buffer I | 800 µl |
| C | Wash Buffer II | 800 µl |
| D | Wash Buffer II | 800 µl |
| E | Elution Buffer D ⁴ | 100 µl |

³ It is important to mix the bottle with MAP Solution A carefully by vigorously shaking or vortexing before use.

⁴ For elution 100 – 200 µl Elution Buffer D can be used.

D. After the program is completed, the tube strip tray is removed from the instrument and the eluates are stored for further use.

Detection of DPYD exon 14 skipping mutation by PCR

Routinely a 2 µl aliquot of each eluate (1/50) is used for the detection of the DPYD exon 14 skipping mutation. The PCR method is developed in-house, based on the sequence entry for chromosome 1p22 gi:88942921. Primers for a PCR fragment of 190 bp were designed. This PCR allows allelic discrimination by means of melting curves.

Results

For detection of the DPYD exon 14 skipping mutation, the shown extraction method (InviMag Blood DNA Mini Kit/ KFmL) can realize highly efficient purification

of genomic DNA from human whole blood. DNA was isolated on the KingFisher mL from 150 µl EDTA blood from a cancer patient who should be treated with the anti-cancer drug 5-FU. Figure 1A shows the amplification of the DPYD exon 14 – PCR product. The red line shows the amplification product from the patient DNA, the green line shows the amplification product from a control DNA of a heterozygote sample. After the detection (Figure 1A), the LightCycler measures a melting curve of the amplified product. Figure 1B shows the first derivation of the melting curve. If the first derivation of the melting curve shows two maxima, a heterozygote DPYD mutation is detected. The patient sample (red line) does not show a mutation and the patient can be treated with a 5-

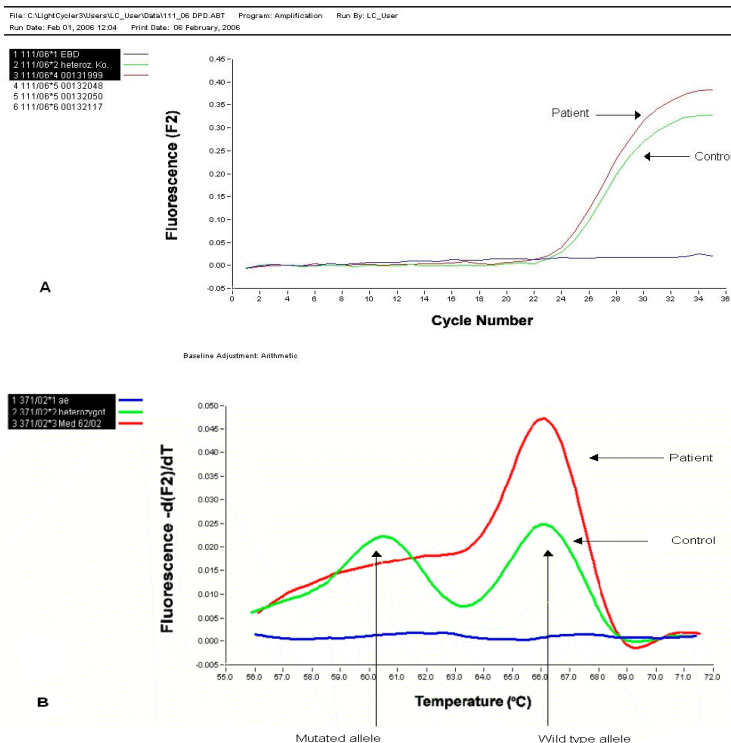


Figure 1: DNA was isolated from 150 µl EDTA blood on the KingFisher mL using the InviMag Blood DNA Mini Kit/ KFmL. 2 µl of the eluated DNA (1/50) was used in a qualitative PCR on a LightCycler (Figure 1A). Figure 1B shows the first derivation of the melting curve of the amplified product.

Red line: patient sample
Green line: heterozygote control

FU standard dose. In the results, a detailed example of a typical DPYD exon 14 skipping mutation analysis is shown. This analysis is performed routinely using the described assay in the clinical laboratory.

Conclusion

New molecular techniques allow a prediction of individual metabolic capacity. These techniques require extremely high efficiency and sensitivity for isolation and detection of nucleic acids. The procedure described above fulfils the required criteria with respect to the mutation screening for the DPYD gene prior to 5-FU treatment in cancer patients. The InviMag Blood DNA Mini Kit/ KfMl provides rapid and economical purification of genomic DNA from blood samples. In combination with the KingFisher mL it has also been successfully used in this laboratory for other diagnostic screenings from blood samples, for example, translocation in chromosome 8,14 or the detection of B-cell tumor disease.

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