# Chapter 5

# Rapid genotyping of the organic anion transporter polypeptide 1B1 polymorphisms A388G and T521C with real-time polymerase chain reaction fluorescence resonance energy transfer assays

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## Abstract

Background and aim

The organic anion transporting polypeptide 1B1 (OATP1B1) polymorphisms A388G and T521C of the solute carrier organic anion-transporter family member 1B1 gene (SLCO1B1), previously known as OATP-C, have potential impact on the drug metabolism.

Materials and methods

In order to set up a fast and consistent assay for these polymorphisms, we developed for both OATP1B1 polymorphisms rapid speed polymerase chain reaction (PCR) fluorescence resonance energy transfer (FRET) assays on the LightCycler.

Results

A locked nucleic acid (LNA) on the polymorphic location within the sensor probe was necessary to discriminate both alleles of the OATP1B1 T521C polymorphism. To confirm the reliability of both real-time PCR FRET assays, these new methods were validated by genotyping 120 samples using a PCR restriction fragment length polymorphism (RFLP) assay and an allele-specific PCR.

Conclusion

The results of the real-time PCR FRET assays were completely in line with the conventional PCR methods, indicating that the real-time PCR FRET assays are appropriate for clinical settings.

# Introduction

The solute carrier organic anion-transporter (SLCO) family, also known as the organic anion-transporting polypeptides (OATP), represent a family of proteins responsible for the membrane transport of a large number of endogenous and xenobiotic compounds. Within this family, OATP1B1, OATP2B1 and OATP1B3 have now been established as the major OATPs located at the basolateral membrane of human hepatocytes. OATP1B1, also known as liver specific transporter-1 (LST-1), OATP-C or OATP2, is involved in the hepatocellular uptake of a variety of endogenous and foreign chemicals[1-4]. Different groups have identified a number of single nucleotide polymorphisms (SNPs) in the SLCO1B1 gene[5,6]. *In vitro* experiments showed that some OATP1B1 allelic variants exhibit a markedly reduced uptake of the OATP1B1 substrates estrone sulfate and estradiol 17 β-D-glucuronide[5,7,8]. Several SNPs introduce amino acid changes in the OATP1B1 protein that result either in altered transport activity or affect the substrate specificity of this protein, as has for instance been shown for the substrates $E_2$17βG and cholyltaurine[5,8,9]. More recently, several publications reported the OATP1B1*1b (388G), OATP1B1*5 (521C) and OATP1B1*15 (388G and 521C) variant alleles, which have relatively high allele frequencies in different ethnic populations and are associated with significantly altered pharmacokinetics of pravastatin, irinotecan and its active metabolite SN-38[10-13]. Until now these OATP1B1 alleles have been genotyped with either a PCR restriction fragment length polymorphism (RFLP) assay (A388G) or with an allele-specific PCR assay (T521C)[5,6]. In order to perform large epidemiological studies or for routine clinical use, a rapid genotype method is preferred. Therefore, the present study describes real-time PCR fluorescence resonance energy transfer (FRET) assays, which are able to genotype these OATP1B1 polymorphisms in considerably less hands on time and with less contamination risk. The results are compared with both a PCR RFLP assay and with an allele-specific PCR assay.

# Materials and methods

## Study design

For the present study genomic deoxyribonucleic acid (DNA) was isolated from 120 anonymized samples originating from a healthy Caucasian population volunteers which consisted of 73 males and 47 females with a median age of 37 years (19-94 years). DNA was isolated from 200 μl ethylene diamine tetra-acetic acid (EDTA) anti-coagulated blood with a High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) or a QIAamp blood mini kit (Qiagen, Leusden, the Netherlands) according to the manufacturers' instructions. Genotyping for the OATP1B1 A388G and T521C polymorphisms was performed using real-time PCR FRET assays

on the LightCycler (Roche Diagnostics, Almere, the Netherlands). A PCR RFLP for the A388G polymorphism and an allele-specific PCR for the T521C polymorphism were used to confirm the results obtained by the real-time PCR FRET assays[6]. The digested and allele-specific PCR products were electrophoresed on a 3% MetaPhor® agarose gel containing 0.3 mg/l ethidium bromide at 75V for 1 hour and 30 minutes, respectively, and examined under ultraviolet illumination. FRET genotyping was also validated by sequencing a homozygote allelic variant sample, a heterozygote sample and a wild type sample of both OATP1B1 polymorphisms. The PCR products were sequenced according to a direct sequence procedure performed with the capillary sequencer ABI 3100® (Applied Biosystems, Fostercity, USA) using the Bridge version 1.1 sequence kit (Applied Biosystems, Fostercity, USA).

## Genotyping for OATP1B1 T521C polymorphism

The primers F27: 5'-gTA gAC AAA ggg AAA gTg ATC ATA -'3 (sense; 80677-700) and R27: 5'-gTT AAA TTT gTA ATA gAA ATg C-'3 (anti-sense; 80936-915) were used to amplify a 260 bp part from the SLCO1B1 gene which covers the T521C polymorphism in exon 5 (Genbank acces no: AC022335). Detection was carried out using the anchor probe OATPCA27: 5'-TAA TAT gCT TCg Tgg AAT Agg ggA gAC TC-'3 (anti-sense; 80823-795) which was labelled at the 5'-end with LCRed 640 and phosphorylated at the 3'-end to block extension and the sensor probe OATPCP27L: 5'-Tgg ATA TAT gCg TTC ATg g-'3 (anti-sense; 80843-825) which is complementary to the OATP1B1 521C polymorphism. This 3'-fluorescein-labelled sensor probe, in which the polymorphic locked nucleic acid (LNA) is underlined, binds with a distance of one base 5' to the detection probe[14,15]. The PCR mixture contained 5.9 μl sterile water; 3.0 mM $MgCl_2$; 2.0 μl LC Faststart DNA Master Hybridization Probes (Roche Diagnostics GmbH, Mannheim, Germany); 0.50 μM of each primer and 0.225 μM of both anchor and sensor probe (TIB MOLBIOL, Berlin, Germany). After adding 2.0 μl containing 50-100 ng genomic DNA to the PCR mixture the total volume is 20 μl. The PCR protocol included the following steps: a) denaturation for ten minutes at 95°C b) 55 cycles at 95°C for five seconds; 51°C for ten seconds and 72°C for 20 seconds. After amplification was completed, a melting curve was recorded by heating to 95°C (20°C/second), holding at 95°C for 20 seconds then cooling to 40°C (20°C/second), holding at 40°C for 20 seconds and then heating slowly to 85°C at 0.2°C/s.

## Genotyping for OATP1B1 A388G polymorphism

The primers F28: 5'-CTC Agg TgA TgC TCT ATT gAg TgA -'3 (sense; 82564-587) and R28: 5'-ggA AAT TgA CAg AAA gTA CTC Tgg T-'3 (anti-sense; 82809-785) were used to amplify a 246 bp part from the SLCO1B1 gene which covers the A388G polymorphism in exon 4 (Genbank acces no: AC022335). Detection was carried out using the anchor probe OATPCA28: 5'-TAC CTg TAA CTg TAA gAA CAT CAC TgA ATT AAA CAT TTT gC-'3 (sense; 82664-704) which was labelled at the 5'-end

with LCRed640 and phosphorylated at the 3'-end to block extension and the sensor probe OATPCP28: 5'-TTT CTg ATg AAT TgA TAT TAg TTT CTT TAg A-'3 (sense; 82632-662) which is complementary to the OATP1B1 388A polymorphism. This 3'-fluorescein-labelled sensor probe, in which the polymorphic nucleotide is underlined, binds with a distance of one base 5' to the detection probe. The PCR mixture contained 6.4 μl sterile water; 3.0 mM $MgCl_2$; 2.0 μl LC Faststart DNA Master Hybridization Probes, 0.50 μM of each primer and 0.20 μM of both anchor and sensor probe. After adding 2.0 μl containing 50-100 ng genomic DNA to the PCR mixture the total volume is 20 μl. The PCR protocol included the following steps: a) denaturation for ten minutes at 95°C b) 45 cycles at 95°C for ten seconds; 55°C for ten seconds and 72°C for ten seconds. After amplification was completed, a melting curve was recorded by heating to 95°C (20°C/second), holding at 95°C for 30 seconds, subsequently cooling to 60°C (0.5°C/second), holding at 60°C for 30 seconds and finally cooling to 45°C for 0 second (20°C/second) and then heating slowly to 75°C at 0.2°C/second. During the slow heating procedure of the melting curves, fluorescence was measured continuously to monitor the dissociation of the fluorophore-labelled detection probe. The fluorescence signals were then converted to melting peaks by plotting the negative derivative of the fluorescence with respect to temperature *versus* temperature (-d(F2/dT *versus* T).

## Results

During the optimalisation of the real-time PCR FRET assay for the OATP1B1 T521C polymorphism, several FRET designs have been tried. For the OATP1B1 T521C assay we used the same primers as Tirona *et al.* for their allele-specific PCR[5]. However, discrimination of the different allelic variants of the OATP1B1 T521C polymorphism was not possible using standard FRET probes. The modified FRET T521C assay applied a sensor probe in which the cytosine on the polymorphic location is substituted by an analogues locked nucleic acid (LNA). Due to their more rigid sugar moiety, LNA bases have a higher binding affinity to their complementary nucleic acids[15]. Hence, shorter sensor probes can be used which makes a better allelic discrimination possible[14]. Because the binding affinity of a well-designed LNA probe is remarkably decreased if hybrids contain one mismatched basepair (bp). For the OATP1B1 T521C polymorphism, the mean melting point ± standard deviation (SD) of the sensor probes was 52.2 ± 0.29°C (n = 82) when hybridized to the OATP1B1 521TT polymorphism and 59.1°C (n = 3) when hybridized to the OATP1B1 521CC polymorphism. The mean difference ± SD between two melting temperatures of 35 heterozygous samples of the OATP1B1 T521C polymorphism was 7.6 ± 0.19°C (Figure 5.1A).

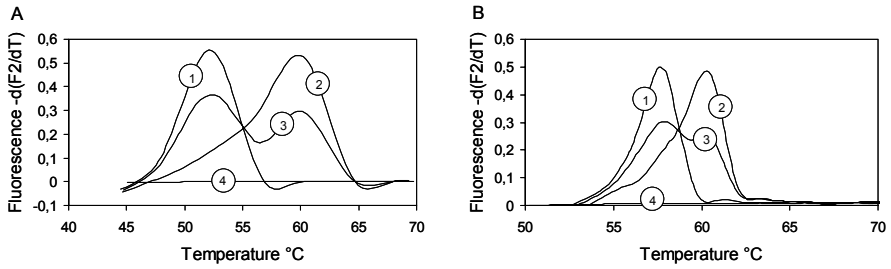


Figure 5.1 Genotyping of the OATP1B1 A388G and T521C polymorphisms with allele specific fluorescent probes by derivative melting curve plots. The –d(F2/dT) derivative melting curves are read in channel 2 for the detection of both OATP1B1 fragments.
A. The derivative melting curve is plotted for a sample homozygous for the 521T allele (1; Tm 52.1°C); a sample homozygous for the 521C allele (2; Tm 59.4°C) and a heterozygous sample (3; Tm 52.3°C and 60.0°C).
B. The derivative melting curve is plotted for a sample homozygous for the 388G allele (1; Tm 57.6°C); a sample homozygous for the 388A allele (2; Tm 60.0°C) and a heterozygous sample (3; Tm 57.9°C and 60.4°C). Melting analysis of a no template control was also performed for both assays (4).

Although we tried to optimise several FRET designs for the OATP1B1 A388G polymorphism, including the use of a LNA in the sensor probe, the discrimination between the melting peaks of heterozygous samples is still limited. Tirona *et al*. used primers for the OATP1B1 A388G assay which have a melting temperature (Tm) that is too low for application in this real-time PCR FRET assay[5]. Because this whole sequence area is very A/T rich, unusual long sensor and anchor probes were necessary to obtain a sufficient high probe Tm[16]. For the OATP1B1 A388G polymorphism, the mean melting point ± SD of the sensor probes was 59.5 ± 0.14°C (n = 43) when hybridised to the OATP1B1 388AA genotype and 57.3 ± 0.14°C (n = 16) when hybridised to the OATP1B1 388GG genotype. This is close, hence 61 heterozygotes do not demonstrate two separated melting peaks. Yet, as is shown in Figure 5.1B, they can be discriminated from either wild type or variant homozygotes. In total, after genotyping 120 individuals in five different PCR runs for both polymorphisms, the frequency of the OATP1B1 521TT genotype is 68%, for the 521CT genotype 29% and for the OATP1B1 521CC genotype 3%. In addition, the frequency of the OATP1B1 388AA genotype is 36%, for the 388AG genotype 51% and for the OATP1B1 388GG genotype 13%. The allele frequencies of the volunteers examined is 17% (521C) and 39% (388G) which is in line with those published in other studies[5,6,10]. To evaluate the reliability of the real-time PCR FRET assays on the LightCycler the 120 samples were also genotyped for the OATP1B1 A388G polymorphism with the PCR RFLP assay using TaqI (Roche Diagnostics GmbH, Basel, Switzerland) and for the T521C polymorphism using an allele-specific PCR[6]. The A388G substitution introduces a restriction enzyme cleavage site for TaqI. Accordingly,

after digestion with TaqI, the fragments of the 388A allele were 90 and 72 bp, whereas the amplicon of the 388G allele is digested into 90, 49 and 23 bp fragments. Individuals carrying both 388 alleles yield 90, 72, 49 and 23 bp fragments. An allele-specific PCR was used for the confirmation of the T521C polymorphism. Depending on the presence of a 521T or C allele a 142 bp fragment appears after electrophoresis of the PCR products. The PCR products and fragments obtained by allele-specific PCR and PCR RFLP were of the expected sizes and the genotypes determined by both methods matched completely with the genotypes determined with the real-time PCR FRET assays. Furthermore, sequence analysis confirmed the results obtained by both real-time PCR FRET assays, the PCR RFLP and the allele-specific PCR.

## Highlights

- Recently, several studies found a correlation between OATP1B1 388G and OATP1B1 521C allelic variants and blood levels of several drugs and/or their metabolites. Individuals carrying one of both OATP1B1 521C alleles eventually in combination with OATP1B1 388G showed significantly higher blood levels.
- Nowadays, genotyping of these polymorphisms is still performed by an allele specific PCR or a PCR RFLP method which are conventional genotyping methods that are time consuming and with a high contamination risk in contrast to the real-time PCR methods.
- In this study we describe two real-time PCR FRET assays on the LightCycler for genotyping the OATP1B1 A388G and T521C allelic variants. The results of both conventional PCR RFLP and allele-specific PCR methods are compared with the results of the real-time PCR FRET assays. The real-time PCR FRET assays are also validated by sequencing a homozygote allelic variant sample, a heterozygote sample and a wild type sample of both OATP1B1 polymorphisms.
- The locked nucleic acid (LNA) on the polymorphic location used in the sensor probe of the FRET assay makes discrimination of the OATP1B1 521C allelic variants possible. Therefore, implementation of a LNA on a polymorphic location in a sensor probe can contribute to applications for FRET probes in real-time PCR assays for polymorphism detection.
- Given the potential impact of functional OATP1B1 polymorphisms on drug metabolism, reliable and fast assays are clearly needed. Therefore, the overall goal of this study is to provide a fast and consistent genotyping method for the OATP1B1 A388G and T521C polymorphisms which can be used in epidemiological studies or routine clinical settings.

## Outlook and conclusions

Since new polymorphisms are being discovered in a high speed, the need for fast and reliable genotyping methods becomes clear. Especially, polymorphisms in genes which have a significant effect on several drug blood levels and with a high allelic frequency, are of special interest. Recently, two functional OATP1B1 polymorphisms were discovered, which have apparently a cumulative effect on pravastatin blood levels. Genotyping 120 volunteers for these OATP1B1 polymorphisms with both real-time PCR FRET assays indicates complete concordance with the genotyping obtained by the PCR RFLP assay for OATP1B1 A388G and the allele-specific PCR assay for OATP1B1 T521C. Based on these results, we conclude that genotyping of the OATP1B1 A388G and T521C polymorphism with a real-time PCR FRET assay on the LightCycler is a very reliable, robust and reproducible technique.

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