Optimized Strategy for Rapid Cytochrome P450 2D6 Genotyping by Real-Time Long PCR

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Background: Because of genetic polymorphisms, cytochrome P450 2D6 (Cyp2D6) activity in humans varies widely and alters the metabolism of commonly used drugs such as antidepressants, neuroleptics, and cardioactive agents. Severe adverse effects or resistance to therapy may result.

Methods: We performed long PCR on the LightCycler™ and used the product as a template for a previously validated multiplex PCR that examines the *3, *4, *6, *7, and *8 alleles of Cyp2D6. We used real-time PCR to identify the *5 null allele and duplication of Cyp2D6 with detection by either hybridization probes or SYBR Green®. The *2 –1584 C/G polymorphism and the *35 allele were identified by PCR with detection by hybridization probes. Products of all PCRs were visualized with gel electrophoresis using a 0.7–1.5% agarose gel and ethidium bromide. Samples containing the *35 allele were analyzed in parallel by digestion with NlaIII, MstI, and BstXI and Smal. We analyzed samples from volunteers and patients (105 samples for deletion and duplication and 116 samples for preamplification). Of those samples, 59 were from depressive inpatients taking part in a trial not yet published.

Results: Identical genotyping results for both real-time and conventional PCR were obtained and verified by gel electrophoresis. Use of long-PCR methods on the LightCycler enabled comprehensive analysis of all relevant polymorphisms of the Cyp2D6 gene in 1 working day with a hands-on time of ~3–4 h.

Conclusions: This is the first description of a successful long-PCR application on the LightCycler and the fastest technique for amplification and specific detection of a PCR product of comparable length. The method appears suitable for large clinical and epidemiologic studies.

The gene cytochrome P450 2D6 (Cyp2D6) encodes the enzyme debrisoquine 4-hydroxylase, which metabolizes many widely used drugs such as various antidepressants, neuroleptics, and cardioactive compounds. The Cyp2D6 gene is highly polymorphic with >70 alleles described to date leading to a wide range in enzymatic activity (1). Extensive metabolizers (EMs), who have normal enzymatic activity, can be distinguished from ultrarapid (ultraextensive) metabolizers (UMs), who have increased activity, and from poor metabolizers (PMs), who have very low or even absent enzymatic activity. Approximately 1–2% of a white Caucasian population are UMs (2–4), whereas 5–10% are PMs. Ten to fifteen percent of Caucasians form the subgroup of intermediate metabolizers (IMs), who have impaired yet residual in vivo function of Cyp2D6 (5). The prevalences of the different types of metabolizers vary in different ethnic and also clinical populations.

Up to 99% of PMs in a Caucasian population can be detected with genetic testing for only six alleles (*3, *4, *5, *6, *7, and *8) (6). Recently, the ~1584 C allele in combination with one nonfunctional allele was reported to be strongly associated with the IM phenotype, whereas ~1584 G in combination with one nonfunctional allele was associated with the EM phenotype. Additional testing for the Cyp2D6*2 –1584 C/G polymorphism could possibly allow the prediction of 50–60% of all IM phenotypes (5). Recently, the Cyp2D6*2 –1584 C variant has been termed Cyp2D6*41 by the CYP Allele Nomenclature Committee, whereas Cyp2D6*2 will be reserved for the Cyp2D6*2 –1584 G promoter type (7).

UMs may suffer from therapeutic failure when subjected to standard doses of Cyp2D6 substrates because of the very rapid metabolic conversion of the applied drugs.
Ultrarapid metabolism can be the result of inheritance of alleles with duplicated or amplified functional Cyp2D6 genes (8). Duplication of Cyp2D6 explains only 10–30% of the UM phenotype (9). A 31G>A polymorphism discovered late in exon 1 (Cyp2D6*35) causes amino acid changes (Val11→Met). This Cyp2D6*35 (Cyp2D6*2B) allele may also play a role in determining the UM phenotype (9), although the exact mechanisms of altering Cyp2D6 enzymatic function are unknown (10).

The need to detect gene deletion (*5 allele) and gene duplication as well as to avoid confusion from two pseudogenes (Cyp2D7 and Cyp2D8) has led to the use of time-consuming long-PCR methods.

To shorten analysis and hands-on time, we developed a long-PCR method on the LightCycler™ system (Roche) in combination with product detection by hybridization probes or SYBR Green® for deletion (*5) and duplication of the functional allele and an established nested multiplex-PCR (6) for examination of the *3, *4, *6, *7, and *8 alleles. We also present a PCR analysis for the *2/*41–1584 C/G polymorphism and for the *35 allele, with detection by hybridization probes on the LightCycler.

Materials and Methods
Primers as well as albumin were purchased from MolBiol. Deoxynucleotide triphosphates (dNTPs) for all reactions were purchased from Roche. All reactions were performed on the LightCycler system from Roche with each reaction described taking place in a separate capillary.

Recommended general measures to avoid contamination problems in PCR were followed closely (11).

Preparation of DNA
Blood samples were obtained from unrelated Caucasian individuals, who gave informed consent for the genetic analysis of their DNA. The EDTA-supplemented blood was extracted with the Genomic DNA Purification Kit (Promega).

Detection of Gene Deletion and Duplication by Hybridization Probes
For detection of gene deletion as well as duplication, the positions and lengths of hybridization probes were optimized with regard to melting temperature and specificity by use of a self-developed software tool and BLAST search (12, 13). One common pair of hybridization probes (termed Rep 1 and Rep 2) was designed (Table 1). A sequence in the second half of the large direct repeats immediately downstream of Cyp2D6 and Cyp2D7 was selected for their position. This sequence was selected because of homology in Cyp-REP-6 (Cyp2D6; GenBank accession no. M33388; positions 8108–8158) and Cyp-REP-7 (Cyp2D7; GenBank accession no. X90926; positions 2279–2329). This sequence is therefore part of Cyp-REP-Dup (Cyp2D6 duplication) in its Cyp2D6 homologous second half (8) and part of Cyp-REP-DEL (14; GenBank accession no. X90927; positions 1819–1869; see Fig. 1). In addition, this sequence includes no known polymorphic sites (8).

Both pairs of primers used (Del-F/Del-R and Dup-F/Dup-R; see Table 1) enclose the repeat unit for the specific detection of Cyp-REP-Dup (8) and Cyp-REP-Del (14) and therefore contain the sequence selected for the hybridization probes.

We used 1.3 U of the Expand Long Template PCR System enzyme mixture and the recommended buffer (Roche) in a final volume of 20 μL. The concentrations of the remaining components of the reaction mixture were as follows: 0.5 μM each of the primers (Del-F and Del-R and Dup-F and Dup-R, respectively; see Table 1), 0.2 μM each hybridization probe, 1.7 mM MgCl₂, 0.3 mM dNTPs, 0.5 g/L albumin, and ~250 ng of DNA. The following cycle program was used: 2 min at 95 °C, followed by 32 cycles, each comprising 10 s at 95 °C and 150 s at 68 °C. The melting curve started at 55 °C and finished at 78 °C with a ramp speed of 0.1 °C/s. Gene deletion and duplication were identified by a maximum decrease of fluorescence at 71.5 °C (Fig. 2).

Detection of Gene Deletion and Duplication by SYBR Green
We also developed an alternative method to detect gene deletion and duplication based on non-sequence-specific detection of PCR products by the DNA binding dye SYBR Green in combination with the designated pairs of primers for the detection of gene deletion and gene duplication (15). SYBR Green® (Molecular Probes) was used in a final dilution of 1:58. The final reaction volume of 20 μL contained 2.1 U of Expand Long Template PCR System enzyme mixture and the recommended buffer (Roche). The concentrations of the remaining components of the reaction mixture were as follows: 0.5 μM each of the primers (Del-F and Del-R and Dup-F and Dup-R, respectively; see Table 1), 0.2 μM each hybridization probe, 1.7 mM MgCl₂, 0.3 mM dNTPs, 0.5 g/L albumin, and ~250 ng of DNA. The following cycle program was used: 2 min at 95 °C, followed by 32 cycles, each comprising 10 s at 95 °C and 150 s at 68 °C. The melting curve started at 55 °C and finished at 78 °C with a ramp speed of 0.1 °C/s. Gene deletion and duplication were identified by a maximum decrease of fluorescence at 71.5 °C (Fig. 2).

Fig. 1. Schematic overview of the Cyp2D genes (without Cyp2D8). The top row represents the genomic organization in the case of gene duplication, the middle row represents the wild type, and the bottom row represents the genomic organization in the case of gene deletion. The large boxes (gray for Cyp2D6 and Cyp2D7; open for the duplicated Cyp2D6) represent the Cyp2D genes and gene variants. The smaller, hatched boxes represent the 0.6- and 2.8-kb direct repeats. The very small 2.8-kb repeats are named Cyp-Rep-7, Cyp-Rep-6, Cyp-Rep-Dup, and Cyp-Rep-Del and depend on the accompanying gene. The arrows represent the positions of the used primers (Del-R and Del-F in the case of gene deletion; Dup-R and Dup-F in the case of gene duplication; see Table 1). The hybridization probes are located within the small open boxes inside the direct repeats (Rep 1 and Rep 2; see Table 1). As can be seen, the hybridization probes are suitable for detecting gene deletion as well as gene duplication. Specificity is achieved by use of different primers.
tively; see Table 1), 2.25 mM MgCl₂, 0.3 mM dNTPs, 0.5 g/L albumin, and ~250 ng of DNA. The following cycle program was used: 90 s at 95 °C, followed by 28 cycles, each comprising 10 s at 95 °C and 150 s at 68 °C. The melting curve started at 55 °C and finished at 97 °C with a ramp speed of 0.1 °C/s. Gene deletion was detected by a maximum decrease of fluorescence at 94 °C (data not shown). Gene duplication was detected by a maximum decrease of fluorescence at 91 °C (data not shown).

AMPLIFICATION OF THE WHOLE CYP2D6 GENE
A third long PCR was performed for amplification of the whole CYP2D6 gene. The product of this reaction was then used as a template for an established multiplex PCR as published by Stüven et al. (6) and for the detection of the *35 allele.

We used 1.5 U of the Expand Long Template PCR System enzyme mixture and the recommended buffer (Roche) together with 0.5 μM each primer (C2D6-F and C2D6-R; see Table 1), 1.7 mM MgCl₂, 0.3 mM dNTPs, and 0.5 g/L albumin in a final volume of 8.5 mL. Approximately 350 ng of DNA was added for each reaction.

The conditions were as described for the detection of gene deletion and duplication using hybridization probes: 1 cycle of 2 min at 95 °C was followed by 32 cycles, each comprising 10 s at 95 °C and 150 s at 68 °C. No melting curve was used because this PCR was performed without the use of hybridization probes or any binding dye.

ANALYSIS OF THE *2 −1584 C/G POLYMORPHISM
For the additional analysis of the *2 −1584 C/G polymorphism, we amplified a 1.9-kb DNA fragment with *2-specific primers. We used a −1584 C-specific hybridization probe (termed C2D6-1584C) in combination with an appropriate anchor probe (termed Anchor-1584) for mutation detection by melting curve analysis (see Table 1).

Position and length were optimized with regard to melting temperature and specificity by use of a self-developed software tool and BLAST search. We used 0.85 U of the Long Range PCR System enzyme mixture and the recommended buffer (Peqlab) in a final volume of 20 μL. The concentrations of the remaining components of the reaction mixture were as follows: 1.2 μM primer Up F 14, 0.5 μM primer 10 B, 0.6 μM both hybridization probes, 0.8 μM of the Expand Long Template PCR System enzyme mixture and the recommended buffer (Roche) together with 0.5 μM each primer (C2D6-F and C2D6-R; see Table 1), 1.7 mM MgCl₂, 0.3 mM dNTPs, and 0.5 g/L albumin in a final volume of 8.5 mL. Approximately 350 ng of DNA was added for each reaction.

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mM MgCl₂, 0.1 mM dNTPs, 0.5 g/L albumin, and ~250 ng of DNA. The following cycle program was used: one cycle of 2.5 min at 95 °C, followed by 32 cycles, each comprising 10 s at 95 °C, 10 s at 64 °C, and 85 s at 68 °C. The melting curve started at 40 °C and finished at 70 °C with a ramp speed of 0.1 °C/s. For the −1584 C allele, the maximum decrease in fluorescence was at 56 °C. For the mutant −1584 G allele, the maximum decrease was at 49 °C (Fig. 3).

ANALYSIS OF THE Cyp2D6*35 ALLELE
Analysis of *35 alleles was also carried out in a separate reaction. Cyp2D6 was first specifically amplified as a 4666-bp fragment by long PCR as described above. The product was diluted and used as a template in the following PCR reaction for detection of the *35 allele. Suitable primers and hybridization probes were designed based on GenBank accession no. M33388. Position and length of hybridization probes were optimized using the same procedure as mentioned above.

LC-DNA Master Hybridization Probes (Roche) were used in a final reaction volume of 20 μL with a MgCl₂ concentration of 1.5 mM. The concentrations of the remaining components of the reaction were as follows: 0.75 μM Cyp31F primer, 0.75 μM Cyp31R primer, 1 μM hybridization probes “Wild-type*35” and “Anchor *35”, 0.5 μg/L albumin, and ~250 ng of the previously amplified product. The following cycle program was used: 1 cycle of 90 s at 95 °C, followed by 26 cycles, each comprising 10 s at 95 °C, 10 s at 58 °C, and 30 s at 72 °C. The melting curve started at 51 °C and finished at 75 °C with a ramp speed of 0.1 °C/s. The maximum decrease in fluorescence for the wild-type allele (31 G) was at 64 °C, and the maximum decrease for the mutant allele (31 A) was at 59 °C (Fig. 4).

All samples were analyzed in parallel by digestion with NlaIII, MslI, and BstX. The fragments were separated in a 0.7% agarose gel and visualized with ethidium bromide.

Results
LONG PCR ON THE LightCycler
Three different amplifications were performed for the long-PCR method on the LightCycler. They identified the deletion and duplication of Cyp2D6 and served as a template for an established multiplex-PCR assay to detect other Cyp2D6 null alleles.

The PCR for gene duplication produced a 3.5-kb product, whereas the product of the PCR for gene deletion was 3.2 kb in length.

ANALYSIS OF Cyp2D6 GENE DELETION AND DUPLICATION BY HYBRIDIZATION PROBES
For real-time analysis of gene deletion and duplication by hybridization probes, we used a melting curve at the end of the PCR program mentioned above. Differentiation between deletion and duplication was enabled by performing the PCRs for deletion and duplication in separate capillaries, using the same hybridization probes but different primers. Melting point temperatures were calculated with the nearest-neighbor model (16).

Products of both PCRs were also visualized with gel electrophoresis on a 0.7% agarose gel and staining with ethidium bromide (Fig. 5). All products had the same lengths as products resulting from established conventional PCRs performed with the same primers for examining gene deletion and duplication (8, 17).

ANALYSIS OF GENE DELETION AND DUPLICATION BY SYBR Green
As an alternative approach to hybridization probes, we performed real-time analysis of gene deletion and duplication with the DNA binding dye SYBR Green. In this method, nonspecific fluorescence resulting from any genomic DNA is interpreted by melting curve analysis. Products were identified by differences in the melting temperatures, which resulted from differences in DNA length. The nearest-neighbor model (16) was used for calculation of melting points. Products were 3.5 and 3.2 kb in length (data not shown).

Gel electrophoresis results for the products of the real-time PCR corresponded completely with gel electro-
RESULTS OF PREAMPLIFICATION PCR ON THE LightCycler

A third long PCR was performed to amplify the whole Cyp2D6 gene and gave a product 4.7 kb in length. No real-time analysis of the product was done.

The product of this PCR was used as a template for the established multiplex-PCR method described by Stüven et al. (6), which detects the *3, *4, *6, *7, and *8 alleles. All probes of the long PCR performed on the LightCycler were validated in this way, and the expected five products could be identified each time by gel electrophoresis (data not shown).

Before using the DNA for the mentioned multiplex PCR, we also visualized the product of the preamplification PCR by gel electrophoresis. Analysis was done on a 0.7% agarose gel with ethidium bromide stain (data not shown). The product was 4.7 kb in length, which corresponds with the findings of Stüven et al. (6).

The three long PCRs, for detection of gene deletion and duplication by hybridization probes and amplification of the whole Cyp2D6 gene, used the same temperature protocol, allowing one combined LightCycler run.

ANALYSIS OF THE *2 −1584 C/G POLYMORPHISM

For additional analysis of the *2 −1584 C/G polymorphism, we used a −1584 C-specific hybridization probe in combination with an appropriate anchor probe for mutation detection by melting curve analysis (Table 1). No fluorescence was detectable with *2-negative templates. Melting temperatures were in complete agreement with the predicted melting temperatures based on the nearest-neighbor model (16). The results of probe analysis by gel electrophoresis, used as an internal control, corresponded with those of products deriving from the established conventional PCR (5).

This method was validated by restriction fragment length analysis. The PCR product of the *2 allele-specific PCR was used as a template for a nested PCR with the mutagenic primer F-Sma and primer R-Sma (Table 1). The resulting 151-bp PCR product was digested with SmaI (New England Biolabs). The fragments were separated by agarose gel electrophoresis and visualized with ethidium bromide. The −1584 G homozygote was diagnosed by a 131-bp fragment (and a small 20-bp fragment not distinguishable from the primers); the −1584 C homozygote PCR product remained uncut (data not shown). For identification of the *2 −1584 C/G polymorphism, all probes gave identical results whether deriving from real-time or conventional PCR.

ANALYSIS OF THE Cyp2D6*35 ALLELE

For detection of the Cyp2D6*35 polymorphism, we used a *35-specific hybridization probe in combination with an appropriate anchor probe for mutation detection by melting curve analysis (Table 1). We obtained a 689-bp fragment.

Melting temperatures were in complete agreement with the calculated melting temperatures based on the nearest-neighbor model (16) (Fig. 4). Sixty samples [51 homozygous for 31 G, 2 homozygous for 31 A, and 7 heterozygous (G/A)] were analyzed with both methods, and the results were in complete concordance (18). With heterozygous templates, both peaks were detectable.

All samples were analyzed in parallel by digestion with NlaIII, MslI, and BstXI, and the fragments were resolved by 2% agarose gel electrophoresis and ethidium bromide staining (data not shown).

RESULTS OF REAL-TIME ANALYSIS OF DIFFERENT PROBES AND ALLELIC DISTRIBUTION

We analyzed different samples with known genotypes with all long-PCR reactions, the multiplex PCR, and the assays for the *2 −1584 C/G polymorphism and the *35 allele.

For method development, we used samples from volunteers and patients after they gave informed consent for the genetic analysis of their Cyp2D6 status. Because we preferred samples with “interesting” genotypes (e.g., Cyp2D6*5), we did not determine the distributions of alleles typical for the general population. A total of 105 samples were analyzed for deletion and duplication, and 116 samples underwent preamplification. The results agreed completely with the known genotypes determined by previously published methods (6, 8, 17). Results were reproduced and confirmed independently by different investigators, who repeatedly analyzed the same samples on the LightCycler.

Of those samples, 59 were from depressive inpatients taking part in a trial not yet published. Distribution of
alleles in this subgroup was random, as the participants were not chosen for their genotypes. The allele frequencies in this population were as follows: *1, n = 53; *2C, n = 14; *2G, n = 30; *4, n = 19; *5, n = 1; *1xN, n = 1. Except of one sample, all samples were also tested for *35: G/G, n = 50; G/A, n = 7; A/A, n = 1.

Discussion
GENOTYPING VS PHENOTYPING
Interindividual genetic differences in Cyp2D6 enzymatic activity impair the metabolism of widely used drugs such as antidepressants, neuroleptics, and cardiovascular agents. Severe adverse effects, variations in therapeutic efficacy, and even total resistance to therapeutic treatment may result.

The reference method for eliminating interindividual pharmacokinetic variation is therapeutic drug monitoring. Not only does it allow the control of genetically based variations in metabolism but also assesses all other factors of influence, such as absorption, distribution, compliance, and others. Therapeutic drug monitoring, however, has been used only for a limited number of drugs. It often requires repeated measurements, and achieving steady state can be time-consuming, depending on the drug involved. Pretherapeutic screening of such drugs may help to identify patients at risk for nonresponse to therapy or adverse reactions attributable to genetically based changes in metabolism before initiation of therapy. This could help in choosing the right drug with the right starting dose. It may also be the rationale for a modified, possibly intensified, monitoring strategy (19, 20).

Until recently, most studies used phenotyping with different probe drugs for prediction of enzyme activity. In clinical situations, however, phenotyping has not been used extensively because of several caveats. It requires the application of test drugs that are often not approved and marketed for therapeutic purposes as well as a rather cumbersome sample collection and biochemical analysis. Because of the interaction with many other clinically used drugs, a washout period is often necessary to obtain meaningful results. This implies an inherent ethical problem because patients seeking medical help for their potentially serious medical conditions must remain untreated for several days (20).

On the other hand, genotyping can be performed from only one blood sample or even more easily obtained material. As opposed to phenotyping, it is completely independent of coadministered therapy and sufficiently specific and sensitive in predicting metabolic capacity. Thus, genotyping of Cyp2D6 may become an important future tool in individually optimized drug therapy as well as in phase III clinical trials of new therapeutics metabolized by Cyp2D6 (19, 21). To be of use in a time-critical pretherapeutic screening program, genotyping methods have to be robust, reliable, and rapid and must include all relevant mutations reported for a specific ethnic population. New relevant mutations are still being discovered, e.g., elucidation of the genetic basis of the IM phenotype recently described by Raimundo et al. (5). More relevant polymorphisms are expected to be discovered in the future, e.g., the genetic basis of UMs not displaying a duplication of the functional gene. Therefore, the inclusion of such new polymorphisms should be easily possible.

The PCR methods described to date are time-consuming and, therefore, not suitable for a regular pretherapeutic screening program. Gene chip technology is promising but still in its infancy, and adapting this technology to new polymorphisms is time-consuming and expensive.

THE ROLE OF REAL-TIME PCR
Real-time PCR, on the other hand, allows for easy adoption of new polymorphisms and possibly provides the best means for pretherapeutic genotyping in a clinical setting at present.

We therefore developed three different long PCRs to detect Cyp2D6 gene deletion and duplication and to produce DNA suitable as a template for a multiplex PCR to detect five other Cyp2D6 null alleles and allele *35. These are some of the most time-consuming steps in conventional Cyp2D6 genotyping.

The advantages of real-time PCR are numerous. The faster PCR methodology and elimination of additional steps to analyze PCR products save time and minimize the risks of DNA contamination. Handling is facilitated, and potentially toxic reagents, such as ethidium bromide stain, are avoided.

PRESENTED METHODS: ADVANTAGES AND LIMITS
One advantage of the proposed PCR method for preamplifying the whole Cyp2D6 gene on the LightCycler is that, although there is no real-time detection, it saves hours of working time in comparison with conventional long PCR. Duplication and deletion can then be detected by either hybridization probes or SYBR Green. Our products and reactions were very stable, minimizing elongation time and providing a common protocol for three different PCR assays.

As explained earlier, hybridization probes were designed to be suitable for detection of both the gene deletion and duplication. This and the fact that we developed identical assays for both PCRs—only the primers differ—simplifies preparation of assays, makes for a more consistent process, and minimizes errors. On the other hand, each reaction must take place in a separate capillary to guarantee differentiation of deletion and duplication.

While developing the methods, we also performed temperature protocols even shorter than those presented in this work. We found them suitable only for DNA of superior quality and not stable enough to function for routine purpose. This is why we recommend, in the case of low-quality DNA templates, extending the elongation time and not to add cycles.

One unresolved problem remains in the design of the
PCR amplifications for the detection of gene deletion and duplication. The absence of deletion and duplication can not be differentiated from a failed PCR reaction. To detect a false-negative result, usually a control reaction of the same gene is included. This strategy was used by Lovlie et al. \( (8) \) for the detection of gene deletion. Adapting this method to detection of \( \text{Cyp2D6} \) gene deletion and duplication complicates the selection of suitable hybridization probes because of the high similarity of both sequences. The use of our probes always produced a “positive” signal. Additionally, the amplification of large products was associated with a high rate of unsuccessful reactions resulting from, vice versa, inhibition of the different reactions taking place at the same time. This problem may be explained by the specific conditions used on the LightCycler, e.g., the very small reaction volumes and the fact that the long-PCR reagents were not fully optimized because of our limited experience with long PCR on the LightCycler. The similar method described recently by Stamer et al. \( (22) \) for the detection of allele \(*5\) has the same problems as discussed above. In addition, Steen et al. \( (14) \) did not include a false-negative control in their assay. To date, only a Southern blot provides clear identification of products. At the moment, quality control must include the assessment of DNA quality, successful amplification of the whole \( \text{Cyp2D6} \) gene \( (4777 \text{ bp}) \), and use of suitable control DNA in every series. Future efforts will be aimed at the optimization of these problems.

Nevertheless, all of our PCR protocols have been compared with reliable, established methods \( (5, 6, 8, 9, 17) \) and have been repeatedly tested with probes of well-known, exact genotype.

Validation of all reactions was done by gel electrophoresis with visualization, and genotyping was done as well by established conventional PCR assays for every DNA probe analyzed on the LightCycler \( (8, 17) \). Results of conventional and real-time PCR were compared and matched in every single probe. Not only did the melting curve analysis correspond to the findings by conventional PCR, but the gel electrophoresis of both PCRs showed products of the same lengths and fluorescence intensities (Fig. 5). Reactions were reproduced repeatedly by several members of our team and showed identical results for every sample.

In addition, we developed an assay that amplified the whole \( \text{Cyp2D6} \) gene, the products serving as a template for a multiplex PCR to detect the \(*3, *4, *6, *7, *8, \) and \(*35\) alleles \( (6) \). We adapted the temperature protocol to that of the PCRs for detecting gene deletion and duplication, working with the hybridization probes, and were thus able to perform all three reactions in one combined LightCycler run. The product of the PCR was separated by gel electrophoresis and visualized and corresponded exactly with the product from the preamplification PCR described by Sttiven et al. \( (6) \). The product also produced the five expected products every time it was used as a template for the multiplex PCR. This demonstrates the stability of the reaction and shows that this adaptation of a long PCR is suitable for routine clinical use.

A preamplification assay using SYBR Green as a DNA-binding dye to detect the PCR product worked but diminished the performance and reproducibility of the multiplex PCR. We therefore chose not to use it in routine clinical conditions.

Lastly, we adapted an established PCR \( (5) \) for the LightCycler by use of hybridization probes to facilitate the detection of the IM phenotype; individuals with this phenotype have residual but noticeably impaired metabolism and comprise \( 10–15\% \) of a Caucasian population.

**Hybridization probes and SYBR Green: a comparison**

Theoretically, the detection of PCR products by hybridization probes is accompanied by higher specificity because successful annealing of primers and probes is necessary for generation of the fluorescent signal. An alternative is non-sequence-specific detection using SYBR Green and melting curve analysis to separate products of different lengths. This method is less expensive than utilizing hybridization probes. Both methods for real-time product analysis allow correct identification of the respective genotypes.

The use of hybridization probes allows combined amplification for analysis of gene deletion and duplication and amplification of the whole \( \text{Cyp2D6} \) gene as preparation for the multiplex PCR.

With SYBR Green, the increase in the number of cycles during amplification led to a dramatic increase in the nonspecific background, complicating genotype analysis. In addition, the use of one common PCR protocol using SYBR Green is not recommended. Therefore, analysis with SYBR Green required two separate PCR runs, thus doubling the time for LightCycler analysis.

On the basis of these results, we recommend the use of hybridization probes rather than SYBR Green for routine screening of gene deletion and duplication.

**Conclusions**

We present a reliable, simple, efficient, and fast long-PCR method. Although interethnic differences in genotype distribution exist, our methods provide sufficient sensitivity to detect the most frequently occurring \( \text{Cyp2D6} \) alleles and to identify the PM, IM, EM, and UM phenotypes in Caucasians \( (5, 6, 8, 9, 17) \). The method enables PCR analysis of \( \text{Cyp2D6} \) in 1 working day, substantially reducing the manual labor. This is in contrast to the \(~2–3\) working days needed when conventional methods are used (estimated \(~9–11\) h for preamplification, \(~10\) h for duplication and deletion, and \(~2–3\) h for determining null alleles). Electrophoresis for sample analysis is completely avoided, reducing the working time again by \(~30\) min per PCR. Compared with published methods for \( \text{CYP2D6}^*35 \) and \(*2/*41\), analysis on the LightCycler saves \(~1.5\) and \(~5\) h, respectively. Use of the described methods in one run,
and including appropriate controls, allows analysis of eight patient samples on one instrument within 1 day. This limits the use of this method for large clinical studies, but it might be appropriate for time-critical screening programs in a clinical setting, where fewer patients per day need rapid genotyping results to plan their therapies.

To our knowledge this is the first description of a successful long-PCR application on the LightCycler as well as the fastest method for amplification and specific detection of a PCR product of comparable length. This method can be adapted to other long-PCR applications.

Recently, a real-time PCR to detect alleles *3, *4, *6, *7, and *8 has been developed (22). It adapts and further develops the allele-specific multiplex PCR described by Stüven et al. (6) on the LightCycler. Here we describe the adaptation of the required preamplification (6) to the LightCycler. We developed a single protocol for this preamplification and for the PCR to detect the *5 allele and gene duplication. Hands-on time has been further minimized by the use of identical hybridization probes for detection of gene deletion and duplication and by use of identical amounts of reagents for both reactions.

By combining the assay described by Stamer et al. (22) with our products from the preamplification PCR and the PCR for detection of gene deletion and duplication, it is now possible to detect the major null alleles in a Caucasian population exclusively using real-time technology in one combined LightCycler run. Together with the pre-existing PCR for detection of alleles *35 and *41/*2, this method makes comprehensive analysis of all relevant polymorphisms of the Cyp2D6 gene possible within 1 working day with use of real-time PCR.

References