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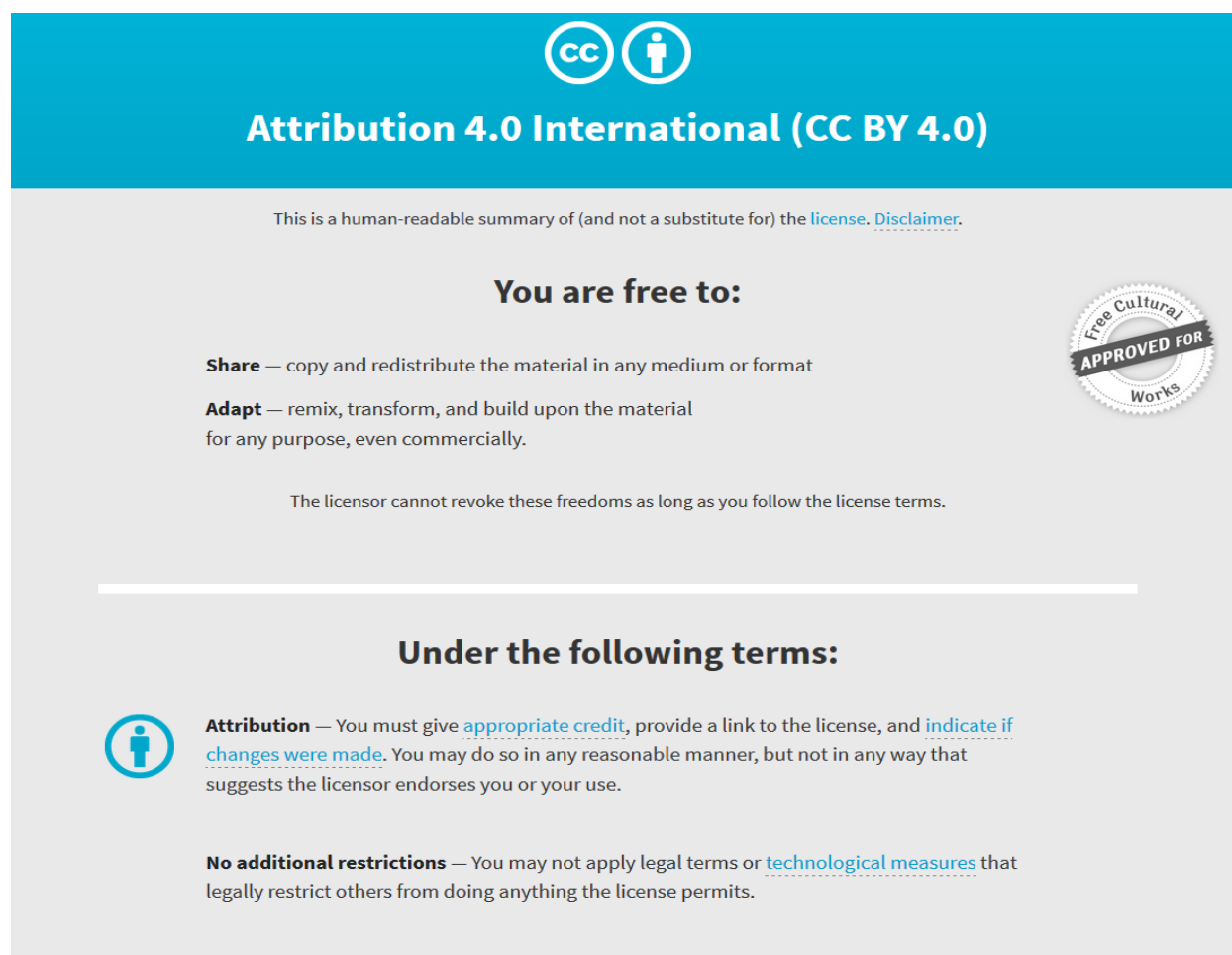
Development of a PCR-based strategy for CYP2D6 genotyping including gene multiplication of worldwide potential use

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Development of a PCR-based strategy for *CYP2D6* genotyping including gene multiplication of worldwide potential use

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*There is growing consensus on the potential use of pharmacogenetics in clinical practice, and hopes have been expressed for application to the improvement of global health. However, two major challenges may lead to widening the "biotechnological gap" between the developing and the industrial world; first the unaffordability of some current technologies for poorer countries, and second the necessity of analyzing all described alleles for every clinical case due to the inability to predict the ethnic group of a given patient. Because of its role in the metabolism of a number of drugs, cytochrome P450 2D6 (CYP2D6) is an excellent candidate for use in the optimization of drug therapy. CYP2D6 is a highly polymorphic gene locus with more than 50 variant alleles, and subjects can be classified as poor metabolizers (PM), extensive metabolizers (EM), or ultrarapid metabolizers (UM) of a given CYP2D6 substrate. Several strategies and methods for CYP2D6 genotyping exist. Some, however, are expensive and laborious. The aim of this study was to design a PCR-based genotyping methodology to allow rapid, straightforward, and inexpensive identification of 90%–95% of CYP2D6 PM or UM genotypes for routine clinical use, independent of the individual's ethnic group. CYP2D6 is amplified in initial extra long PCRs (XL-PCRs), which subsequently undergo fragment-length polymorphism analysis for the determination of carriers of CYP2D6 allelic variants. The same XL-PCRs are also used for the determination of CYP2D6 multiplication and 2D6*5 allele (abolished activity). The application of this new strategy for the detection of CYP2D6 mutated alleles and multiplications to routine clinical analysis will enable the PM and UM phenotypes to be predicted and identified at a reasonable cost in a large number of individuals at most locations.*

INTRODUCTION

Cytochrome P450 2D6 (CYP2D6) is of great importance for the metabolism of clinically used drugs, of which it metabolizes about 20%–25% (1), including antipsychotics, antidepressants, antiarrhythmics, β blockers, etc. (2,3). Recently we have shown that the number of *CYP2D6* active genes is related to the dose-corrected plasma concentration of the widely used drugs thioridazine (4), risperidone (5), and fluoxetine (6) and to the QTc interval, and thus, potentially to the risk of cardiotoxicity in patients (4,7).

CYP2D6 is a highly polymorphic gene locus with more than 50 variant alleles that lead to a widely ranging enzymatic activity (www.imm.ki.se/CYPalleles/cyp2d6.htm). The polymorphism of the enzyme results in poor metabolizers (PM), efficient metabolizers (EM), or ultrarapid metabolizers (UM) of *CYP2D6* substrate drugs. Subjects with multiple gene copies (UMs) will metabolize drugs more rapidly, and therapeutic plasma levels will not be reached at ordinary drug dosages. Individuals lacking functional *CYP2D6* genes (PMs), however, will metabolize selective *CYP2D6* substrates at a slower rate (8) and may thus be more prone to adverse effects due to elevated drug plasma levels. In PMs, there may also be a reduced effectiveness of drug therapy when pro-drugs requiring activation by *CYP2D6* are used.

The variant *CYP2D6* alleles can be classified into categories according to whether they cause abolished, decreased, normal, or increased activity. Although more than 50 different allelic variants have been identified, analyses of *CYP2D6**3, *CYP2D6**4, *CYP2D6**4 duplication, *CYP2D6**5, and *CYP2D6**6 mutant alleles (abolished activity), and gene duplications of *CYP2D6**1 and *CYP2D6**2 (increased activity) have to be performed to allow a 99% sensitive prediction of PMs or UMs in clinical routine among White European and White American populations (9). The *CYP2D6**10 allele is particularly common among Chinese and Japanese populations and is associated with decreased *CYP2D6* activity (10). Furthermore, the presence of the *CYP2D6**17 allele among African populations also shows decreased *CYP2D6* activity (11). Since, with the mapping of the human genome, the concept of race has

become untenable, and given the general increase in admixture of people around the world, there is a need for genotyping for the most frequently identified alleles in a given clinical situation, independent of the location of the ethnic group. In particular, the *CYP2D6* allele frequency varies between populations and geographical areas (9), so that in admixed populations (e.g., in North, Central, and South America), it would be very useful to have a genotyping method that would allow the phenotype of a given patient or healthy volunteer to be predicted.

The general use of *CYP2D6* genotyping may be of help to increase the use of drug therapy and, hence, of global health (12,13). However, most of the several new strategies and methods for *CYP2D6* genotyping, such as single-strand conformation polymorphism (SSCP) (14,15), real-time PCR (16,17), microarrays for DNA analysis (18,19), and TaqMan® real-time PCR (20), are expensive or labor-intensive. This situation may widen the "biotechnological gap" between developed and undeveloped countries.

There have been calls for the use of biotechnologies and pharmacogenetics for improved health in developing countries (12,13). One limitation, however, is the unaffordability of some of the current diagnostic methods. PCR is an affordable molecular diagnostic technology and is already in use in low income regions. The development of a strategy for *CYP2D6* genotyping covering the most commonly described alleles could therefore promote the use of these technologies in the developing world. The aim of this study was to design a genotyping method based on extra long PCR (XL-PCR) and PCR restriction fragment-length polymorphism (PCR-RFLP) methods to allow rapid, straightforward, and inexpensive identification of 90%–95% of *CYP2D6* PM or UM genotypes in a clinical routine, independent of the individual's ethnic group.

MATERIALS AND METHODS

For *CYP2D6* genotyping, 10-mL blood samples were collected in EDTA tubes, and DNA was extracted using the QIAamp® DNA blood kit (Qiagen, Hilden, Germany).

Table 1. PCR Primer Sequences

Primers	Sequences	References
DPKup	5'-GTTATCCCAGAAGGCTTTCAGGCTTCA-3'	21
DPKlow	5'-GCCGACTGAGCCCTGGGAGGTAGGTA-3'	21
2D6dupl-F	5'-CCTGGGAAGGCCCATGGAAG-3'	22
2D6dupl-R	5'-CAGTTACGGCAGTGGTCAGCT-3'	22
5'2D6dup	5'-GCCACCATGGTGTCTTTGCTTTCCTGG-3'	23
3'2D6dup	5'-GGTTTCTTGGCCCCGTGTCCCACTC-3'	23
5'2D6*5	5'-CACCAGGCACCTGTACTCCTC-3'	24
3'2D6*5	5'-CAGGCATGAGCTAAGGCACCCAGAC-3'	24
5'2D6*3	5'-GGTCAGTGGTAAGGACAGGCAGGCC-3'	25
3'2D6*3	5'-TCTCGGGGGGCTGGGCTGGGTCCAGTTGGCC-3'	25
5'2D6*4	5'-TGCGCAACTTGGGCTGGGCAAGAAGTCTGGACCCG-3'	25
3'2D6*4/*6	5'-CTCGGGAGCTCGCCTTGCAGAGACTC-3'	25
5'2D6*6	5'-TGCGCAACTTGGGCTGGGCAAGAAGTCTGGACTAG-3'	25
5'2D6*10	5'-CCTGATGCACCGGCCAACGCTGGGCTGCACAGTAC-3'	25
3'2D6*10	5'-CAAACCTGCTTCCCTTCTCAGCC-3'	25
5'2D6*17	5'-GGCGAGGACACCGCCACCGCCCGCTGTGCCAGTA-3'	25
3'2D6*1×N	5'-CCTGTCTCAAATGCGGCCAGGCGTGGGTA-3'	25

Determination of *CYP2D6* Gene Duplication

To determine whether individuals were carrying duplicated *CYP2D6* genes, XL-PCR was used to amplify a fragment spanning the potential crossover sites. The forward primer (2D6dupl-F) is specific for *CYP2D6* 3' flanking sequences, and the reverse primer (2D6dupl-R) is specific for a *CYP2D7* sequence (21). In addition, in the same PCR, the entire *CYP2D6* gene was amplified by using forward (DPKup) and reverse (DPKlow) primers (22) (see Table 1). Amplification reactions of 25 μ L were performed on a Mastercycler® 384 (Eppendorf AG, Hamburg, Germany) in 0.2-mL thin-walled tubes by using 0.375 μ L of an enzyme blend of *Taq* and *Pwo* DNA polymerases (Expand™ Long Template PCR System; Roche Diagnostics GmbH, Mannheim, Germany). Amplification of 50–100 ng/ μ L human genomic DNA was done using 2.5 μ L PCR buffer 3 (2.75 mM MgCl₂; Expand Long Template PCR System), 0.5 mM each dNTP (Deoxynucleoside Triphosphate Set PCR Grade; Roche Diagnostics GmbH), and 0.4 μ M each primer. The cycling conditions were as follows: 2 min denaturation at 94°C, then 10 cycles of 95°C for 20 s, 68°C for 4 min, then 20 cycles of 95°C for 20 s, 68°C for 4 min (increasing 5 s/cycle), and then a final extension step of 7 min at 68°C. The PCR product was then analyzed directly by 0.8% agarose gel electrophoresis, and the DNA was visualized with ethidium bromide. This XL-PCR generated *CYP2D6* fragments of 5.1 kb, which subsequently served for PCR-RFLP diagnostics, and 3.5 kb if the multiplication allele was present (Table 2).

Detection of the *CYP2D6**5 Allele

To determine whether individuals were carrying *CYP2D6**5 allele genes, XL-PCR was performed. The 5'2D6*5 and 3'2D6*5 primers (23) are specific to identify the presence of the *CYP2D6**5 allele (3.5 kb). In addition, in the same PCR, the entire *CYP2D6* gene (5.1 kb) was amplified as an internal control of the reaction by using forward DPKup and reverse DPKlow primers (22) (Table 1). The amplification reactions, cycling conditions, and DNA analysis were the same as used in the determination of *CYP2D6* gene duplication.

Detection of the *CYP2D6**3, *4, *6, *10, and *17 Alleles

Subsequent nested PCRs were performed to identify the presence of the *CYP2D6**3, *4, *6, *10, and *17 alleles. All reamplifications were carried out in 25- μ L reactions containing 0.5 U EcoTaq polymerase (Ecogen, Barcelona, Spain), 1 μ L *CYP2D6* template (XL-PCR diluted with water 1:10), 2 μ M each primer (see Table 1), 0.2 mM each dNTP, and 1.2 mM MgCl₂. The cycling conditions were: 1 min denaturation at 94°C, then 30 cycles (except the 2D6*6 assay, which has 20 cycles) of 94°C for 10 s, 65°C for 20 s, 72°C for 20 s, and then a final extension step of 7 min at 72°C.

Subsequently, 8 μ L PCR were digested with 5–10 U restriction enzyme (New England Biolabs, Beverly, MA, USA) (see Table 2), followed by an overnight incubation to attain complete digestion. The PCR product was then analyzed directly by 3% agarose gel electrophoresis. The DNA was visualized with ethidium bromide. Table 2 summarizes all the reamplification reactions performed.

Detection of the *CYP2D6**1, *2, or *4 Duplication Alleles

All DNAs possessing gene duplications were further characterized for a *CYP2D6**1×N, *2×N, and *4×N alleles using 1 μ L XL-PCR template (diluted with water 1:10) producing 10 kb. This XL-PCR was performed using the primers 5'2D6dup and 3'2D6dup (Table 1). Amplification reactions of 25 μ L were performed in 0.2-mL thin-walled tubes using 0.375 μ L of an enzyme blend of *Taq* and *Pwo* DNA polymerases. Amplification of 50–100 ng/ μ L human genomic DNA was done using 2.5 μ L PCR buffer 2 (2.75 mM MgCl₂; Expand Long Template PCR System), 0.5 mM each dNTPs (Deoxy-

Table 2. Amplification and Reamplification Reactions Performed for the Determination of *CYP2D6* Variant Alleles

Forward Primers		Reverse Primers		Results (bp)	
DPKup 2D6dupl-F		DPKlow 2D6dupl-R		2D6 gene: 5100 *1, *2, or *4×N: 3500	
DPKup 5'2D6*5		DPKlow 3'2D6*5		2D6 gene: 5100 *5: 3500	
5'2D6dup		3'2D6dup		*1, *2, *4×N: 10,000	
Forward Primers	Reverse Primers	Nucleotide Identified	PCR (bp)	Restriction Enzyme	Results (bp)
5'2D6*3	2'D6*3	2637 (A del)	259 or 258	<i>Stu</i> I	wt: 227 + 32 *3: 258
5'2D6*10	3'2D6*10	188 (T)	241	<i>Scal</i>	wt: 241 *4, *4×2, or *10: 206 + 35
5'2D6*4	3'2D6*4/*6	1934 (G)	242	<i>Pst</i> I	wt: 208 + 34 *4: 242
5'2D6*6	3'2D6*4/*6	1795 (T del)	385 or 384	<i>Spe</i> I	wt: 351 + 34 *6: 384
5'2D6*17	3'2D6dup	1111 (C)	206	<i>Rsa</i> I	wt: 170 + 36 *17: 206
5'2D6dup	3'2D6*1×N	4268 (G)	247	<i>Ban</i> I	*2×2: 214 + 33 *1×2: 247

wt, wild-type

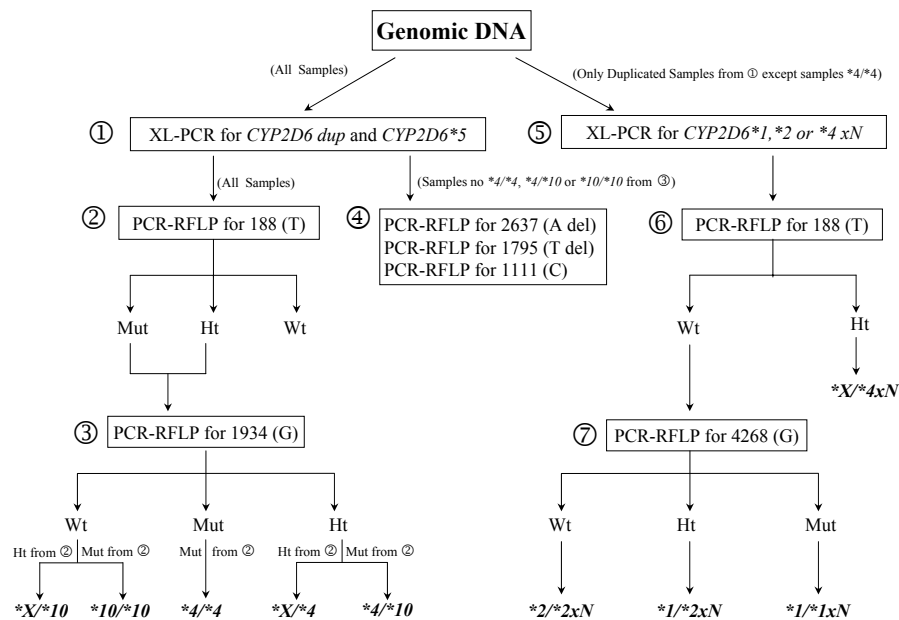


Figure 1. Design of the *CYP2D6* genotyping method. The circled numbers represent the order of the steps of the procedure. The PCR restriction fragment-length polymorphism (PCR-RFLP) in step 6 will produce only Wt or Ht results because it has not included samples *4/*4 with multiplications. Wt, homozygous with no mutations; Het, heterozygous; Mut, homozygous mutated. *X; *CYP2D6* variant allele other than *4 or *10.

nucleoside Triphosphate Set PCR Grade), and 0.3 μ M each primer (see Table 1). The cycling conditions were as follows: 2 min denaturation at 94°C, then 10 cycles of 95°C for 20 s, 59°C for 30 s, 68°C for 9 min 30 s, then 20 cycles of 95°C for 20 s, 59°C for 30 s, 68°C for 9 min 30 s (increasing 10 s/cycle), and then a final extension of 11 min at 68°C. The PCR product was then analyzed directly by 0.8% agarose gel electrophoresis, and the DNA was visualized with ethidium bromide. This XL-PCR generated a fragment of 10 kb, which subsequently served for PCR-RFLP diagnostics. Then reamplifications were carried out to detect the *CYP2D6**1xN, *2xN, and *4xN alleles. Table 2 summarizes all the reamplification reactions performed.

RESULTS AND DISCUSSION

The methodological procedure for the detection of *CYP2D6* mutated alleles and multiplications are summarized in Figure 1. Genomic DNA is amplified in an initial XL-PCR (Figure 1, step 1) for the determination of *CYP2D6* multiplication and the *2D6**5 allele, with each reaction containing two sets of tetraprimers (Table 2). One generated a *CYP2D6* fragment of 5.1 kb, which subsequently served for further PCR-RFLP diagnostics (Figure 1, steps 2–4). The other pair generated 3.5-kb fragments if the *2D6**5 deletion or the *2D6**1, *2, or *4 gene duplication was present. Then, the assays for positions 188 and 1934 were performed to determine carriers of *2D6**4 and *2D6**10 alleles (Figure 1, steps 2 and 3). Subsequently, PCR-RFLP analyses were performed to identify the *2D6**3, *2D6**6, and *2D6**17 variant alleles (Figure 1, step 4).

All DNA possessing a *2D6**1, *2, and/or *4 multiplied allele were amplified (except DNAs homozygous for the *2D6**4 allele, because the multiplication is on this allele) producing a 10-kb fragment (Figure 1, step 5), which was amplified for further reamplifications (Figure 1, steps 6 and 7) and to identify which allele carried a duplication. PCR techniques are not capable of differentiating between a gene duplication or multiplication, and for these individuals, a Southern blot analysis needs to be performed to determine the number of *CYP2D6* genes present.

While there are several strategies and methods for *CYP2D6* genotyping, such as SSCP (14,15), real-time PCR (16,17), microarrays for DNA analysis (18,19), and TaqMan real-time PCR (20), they are expensive and hence unaffordable in many locations. The XL-PCR and PCR-RFLP procedures for *CYP2D6* genotyping described in the present study allow the rapid, straightforward, and inexpensive identification of 90%–95% of clinically relevant *CYP2D6* genotypes based on an already existing biotechnological method and are suitable for worldwide use.

Conclusions

CYP2D6 genotyping is a useful tool in clinical medicine. However one of the problems facing its routine use is its cost in terms of labor, equipment, and reagents, especially in admixed populations where a potentially large number of alleles are present. The application of the present PCR-based method to routine clinical analysis will enable PM and UM phenotypes to be

predicted and identified at a reasonable cost in a large number of individuals covering all ethnic groups. Thus, it might contribute to narrowing the “biotechnological gap” between the industrial and the developing worlds.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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