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CYP2A6 genotyping methods and strategies using real-time and endpoint PCR platforms

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Abstract

CYP2A6 genotyping is of clinical importance *CYP2A6* gene variants influence nicotine metabolism and are associated with nicotine dependence, cigarettes per day, smoking cessation and the risk for tobacco-associated cancers. *CYP2A6* gene variants also influence the metabolism of therapeutic drugs, such as the anti-cancer agents tegafur and letrozole. Over the years, *CYP2A6* genotyping methods have evolved to incorporate novel gene variants and to circumvent genotyping errors resulting from the high degree of homology between *CYP2A6* and neighboring *CYP2A* genes. Herein, *CYP2A6* genotyping strategies are described for commonly genotyped functionally significant alleles including single nucleotide polymorphisms, small insertions/deletions and more complex structural variants. The methods presented utilize higher throughput SYBR green real time polymerase chain reaction technology in addition to standard thermocycling.

Keywords

CYP2A6; real time PCR; endpoint PCR; SNP; hybrid allele; gene deletion

Background

CYP2A6 genotyping has contributed to the understanding of inter-individual differences in the metabolism of nicotine and other therapeutics, such as the anti-cancer agents tegafur and letrozole, as well as smoking behaviors and the risk for tobacco-associated cancers (reviewed in [1–4]). More recently, *CYP2A6* genotype has been established as a significant

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risk factor for lung cancer among Caucasian, African American and Japanese smokers [5–8], and CYP2A6 enzymatic activity, which is influenced by genetic polymorphisms, has been investigated as a tool for optimizing the selection of smoking cessation pharmacotherapy [9–11].

The *CYP2A6* gene is located on chromosome 19q13 within a cluster of *CYP2* family genes (*CYP2T2P*, *2F1P, 2A6, 2A7, 2G1P, 2A18PC, 2B7P, 2B6, 2A18PN, 2G2P, 2A13, 2F1, 2T3P, 2S1*), which share a high degree of sequence homology and are thought to have arisen from a single locus through duplication events [12]. To date, 45 *CYP2A6* star (*) alleles have been identified and listed in the Human Cytochrome P450 Allele Nomenclature Database [13]; additional polymorphisms continue to be identified and characterized [14]. Polymorphisms include single nucleotide polymorphisms (SNPs), small deletions/insertions, gene deletions and duplications, gene hybrids and gene conversions, which affect CYP2A6 enzymatic activity through diverse mechanisms from altering promoter activity to transcript stability and cofactor binding [13, 15]. Importantly, the prevalence of individual alleles varies greatly across world populations contributing to population differences in CYP2A6 activity [16, 17].

The challenge of *CYP2A6* genotyping lies predominantly in the high degree of sequence homology between *CYP2A6* and neighboring *CYP2A* genes (e.g. *CYP2A6* and *CYP2A7* share 95% sequence homology) and to the presence of many structural variants typically requiring more labor-intensive methods to ensure accurate genotype calls [18–20]. Owing to the complex genetic architecture of variation in *CYP2A6* and to the prevalence of large numbers of low frequency alleles, studies using SNP platforms, such as genome wide association studies, have had limited utility identifying significant associations [21, 22]. The increasing number of identified functional variants, often present at low frequencies, also adds to the burden of a genotyping project [14]. To circumvent errors resulting from homology, many assays employ a two-step polymerase chain reaction (PCR) genotyping approach in which a *CYP2A6* gene-specific first PCR amplification is performed and then utilized as template for an allele-specific second PCR amplification. Allele calls are typically based on genotyping the ‘defining/functional’ SNP or insertion/deletion, or, in the case of a hybrid allele, the crossover point, as opposed to typing the entire haplotype, which may include numerous additional synonymous or intronic alterations in linkage disequilibrium with the ‘causal’ variant [13], as time, cost and limited DNA are important considerations and high through-put sequencing for this gene is not yet available. In support of this approach, there is agreement between genotype-phenotype associations *in vivo* and the relationship predicted from investigations of ‘defining’ SNPs and variants *in vitro* [14, 23–25]. A limitation of this genotyping approach is that individuals may harbour additional variants, which were not screened for, or may not harbour all of the variants as defined in the *CYP2A6* nomenclature database.

Herein, PCR-based methods and strategies (i.e. Method 1: Endpoint PCR allele amplification, and Method 2: SYBR green allele amplification) are presented to genotype alleles with altered activity that are found at appreciable frequencies in at least one racial/ethnic populations, *CYP2A6*1B, *2, *4, *9, *12* and *1X2A*, found predominantly in populations of Asian descent, *CYP2A6*7, *8* and *10*, and found predominantly in

populations of African descent, *CYP2A6*17, *20, *23–28, *31*, **35*, and **1X2B* alleles [16, 17, 26]. Assays for the *CYP2A6*34* allele are also included – genotypes with this allele are associated with reduced *CYP2A6* activity, however the allele has not been extensively genotyped across populations (unpublished findings and [27]). With respect to nicotine metabolism, the *CYP2A6*2*, **4*, **7*, **17* and **20* alleles are considered inactive toward nicotine [17, 24, 25, 28–34], the *CYP2A6*9* and **12* alleles have decreased nicotine metabolic activity whereby *CYP2A6 *1/*9* and **1/*12* individuals have about 75% of the CYP2A6 activity and 80% of the total nicotine clearance of *CYP2A6*1/*1* individuals [33, 34], the *CYP2A6*23-*28,* and **35* alleles also have evidence of reduced CYP2A6 metabolic activity towards nicotine [23, 26, 35, 36], and the *CYP2A6*1B* and *CYP2A6*1X2* duplication alleles have modestly increased activity [20, 37, 38].

Traditional two-step PCR genotyping assays using endpoint PCR and gel electrophoresis to visualize results are described for all of the alleles mentioned above (Method 1). Additionally, for *CYP2A6*1B, 9*, **12*, **17*, **20*, **23*, **24*, **31*, **34* and **35*, two-step PCR genotyping assays using the real time PCR platform and SYBR green to visualize results are provided (Method 2). *CYP2A6* genotyping assays using real time PCR and SYBR green expedite the genotyping of large sample sets and minimize the use of genomic DNA, often a precious resource. Aside from the PCR-based methods outlined in this report, a number of commercial TaqMan, SNAPshot and array-based assays are available (e.g. those from Thermo Fisher Scientific, Illumina's ADME panel, Affymetrix' DMET panel), with the caveat that these generally work best for SNP genotyping in regions of limited homology. The only assay validated in the authors' laboratory, is the TaqMan assay (Thermo Fisher Scientific) for the *CYP2A6*2* allele (unpublished data). Commercial TaqMan assays, which we have not validated, are also available for the *CYP2A6*17* and **20* alleles and to asses copy number variation.

General genotyping strategies for *CYP2A6* alleles defined by SNPs or small indels

The general strategy for alleles that are defined by either a SNP or a small insertion or deletion, such as *CYP2A6*2, *7, *8, *9, *10, *17, *20, *23-*28, *31, *35* (Figure 1) is a nested PCR approach whereby the first amplification, which is gene-specific, serves as a template for an allele-specific second amplification (Method 1.1 and Method 2.1). The allele-specific amplifications are designed around a 'defining/functional' SNP or indel within each allele – for example, the *CYP2A6*2* second amplification targets the T>A SNP at genomic position 1799 (rs1801272), which results in the Leu160His amino acid substitution and loss of heme binding [19, 30]; *CYP2A6*9* targets the T>G SNP at genomic position −48 (rs28399433), which disrupts the TATA box and results in reduced transcription [39, 40]; while *CYP2A6*20* targets the deletion at genomic position 2141–2142, which causes a frameshift that results in an inactive truncated protein [24]. Similarly, allele-specific amplifications for *CYP2A6*17* (rs28399454), **23* (rs56256500), **28* (rs8192730) and **31* (rs72549432) target SNPs resulting in amino acid substitutions [25, 35, 36]. For alleles, such as *CYP2A6*10*, **24*, **26* and **27*, which are defined as a haplotype of multiple SNPs/indels that may occur either separately or together, haplotypes can be tested directly or inferred

based on the co-occurrence of variants in the same individual. For example, the *CYP2A6*10* haplotype, which can be inferred based on the presence of the G>T SNP at position 6600 (also known as *CYP2A6*8*, rs28399468) among those with *CYP2A6*7* (rs5031016), can also be determined directly through a haplotyping assay (described in Method 1.1) [28, 41]. Likewise, the *CYP2A6*26* and **27* haplotypes can be inferred based on the co-occurrence of the T>G SNP at position 1711 (rs59552350) or the GC>A indel at 2162-3 (rs28399445), respectively, with *CYP2A6*25* (rs2839940) [36]. Similarly, the *CYP2A6*24* haplotype can be inferred based on the G>C SNP at genomic position 594 (rs72549435) co-occurring with *CYP2A6*35* (rs61736436) [36].

General genotyping strategies for *CYP2A6* alleles defined by structural variation

In addition to SNPs and indels, many structural variants have been identified in the *CYP2A6* locus: the **1B* gene conversion [42], the **4* gene deletions [29], the **1x2* gene duplications [20, 38] and the **12* and **34* hybrid alleles [27, 43] – each of these are hypothesized to result from unequal crossover events between *CYP2A6* and the highly homologous *CYP2A7* pseudogene during recombination [29, 38, 43]. Nested endpoint PCR assays are employed to genotype these structural variants. The first amplifications employed in these genotyping assays target upstream and/or downstream sequences shared by *CYP2A6* and *CYP2A7*, whereas the second amplifications typically target the *CYP2A6-CYP2A7* crossover region. For example, the *CYP2A6*4* alleles are a family of *CYP2A6* deletion alleles, each with a different amount of the remnant of the *3′* end of *CYP2A6* appended to *CYP2A7* (Figure 2A). The first amplification detects either wild-type *CYP2A6* or the deletion allele using a *5′* primer that anneals to either *CYP2A6* or *CYP2A7* and a *3′* primer specific for *CYP2A6* (Figure 2B & 2D), while the second amplification distinguishes between the wild-type and deletion allele (Figure 2C & 2E).

Method 1: Endpoint PCR allele amplification

Genotyping assays using endpoint PCR and gel electrophoresis to visualize results are described. All assays employ a two-step PCR genotyping approach in which the first PCR amplification is utilized as template for an allele-specific second PCR amplification that targets either the 'defining/functional' SNP or indel (Method 1.1), or, in the case of a hybrid allele, the crossover point (Method 1.2). Over the years as novel genetic variation was discovered which confounded original genotyping strategies (e.g. SNPs underneath first or second amplification primers), many primers were redesigned [29, 36, 37], and references to original or previous versions of primers and genotyping assays are provided (Supplementary Table 1) and a note on the rationale for recent primer modification is provided (Supplementary Table 2).

1.1. Protocols for *CYP2A6* SNPs & small indels

First and second amplification primers for the *CYP2A6*2, *7, *8, *9, *10, *17, *20, *23-*28, *31, *35* endpoint PCR genotyping assays are presented (Table 1 for primer combinations, Supplementary Table 3 for primer sequences). As indicated (Table 1),

typically 2–4 allele-specific second amplifications use a common first amplification as template requiring ~250 ng of genomic DNA per sample to genotype all 15 SNP/indel alleles. The assays presented in Methods 2.1 reduce the genomic DNA requirement by utilizing two common first amplifications for all SNP/indel allele-specific assays. Reaction mixtures, cycling and gel electrophoresis conditions are provided (Table 2A and Table 2B).

1.2. Protocols for *CYP2A6* structural variants

First and second amplification primers for the *CYP2A6*12* and **34* hybrid alleles, **1B* gene conversion, **4* gene deletions, and **1X2A*, **1X2B* gene duplications endpoint PCR genotyping assays are presented (Table 1 for primer combinations, Supplementary Table 3 for primer sequences). Reaction mixtures, cycling and gel electrophoresis conditions are provided (Table 2). *CYP2A6*12* and **34* comprise *CYP2A7* sequence from exon 1 to introns 2 and 4, respectively, and *CYP2A6* sequence downstream of those crossover points, and a common first amplification is provided as template for both allele specific assays. *CYP2A6*1B* contains a *CYP2A7* gene conversion in the 3′ untranslated region of *CYP2A6*, which is associated with increased transcript stability but does not alter the enzymatic structure of CYP2A6 (essentially a different version of the **1* "wild-type" allele) [37, 42, 44]. As many reduced/loss of function *CYP2A6* SNPs (e.g. **7*, **8*, **10*, **24*, **28*, **35*) occur in haplotype with this gene conversion [13], it is advisable to restrict **1B* genotype-phenotype analyses to those subjects without other *CYP2A6* variants [37]. Multiple *CYP2A6*4* deletion assays exist, as additional crossover points have been discovered between *CYP2A6* and *CYP2A7* [29]. The *CYP2A6*4H* assay detects all of the known **4* deletion alleles (i.e. **A*, **D-*H*) except for the **4B* deletion allele, which has a crossover point *3′* of **4H* [29, 45]. The *CYP2A6*4H* first amplification is relatively long at 3,538 base pairs, and call rates can be improved from genomic DNA samples of lower quality using Pfu versus Taq DNA polymerase (Table 2A). An alternative genotyping approach for those exclusively interested in differentiating the *CYP2A6*1B* and **4C* alleles from "wild-type" is a single PCR amplification of the 3′ end of *CYP2A6/CYP2A7* followed by digestion with two restriction enzymes [42]. Assays have been developed for two gene duplication alleles, *CYP2A6*1X2A* and **1X2B,* which are believed to be the reciprocal products of the unequal *CYP2A6-CYP2A7* crossover events that created the **4D* and **4B* deletion alleles, respectively [20, 38].

Method 2: SYBR green allele amplification

The real time platform offers immediate time-savings over traditional endpoint PCR by eliminating the need for gel electrophoresis to visualize results. The use of SYBR green to detect amplification has the advantage of ease and low cost of assay modification with the identification of novel genetic variants, which likely will continue to be found within primer sequences. TaqMan technology requires the use of both specialized primers and probes, which may be more costly to modify. A nested PCR strategy is utilized in the real time genotyping protocols to mitigate the high homology between *CYP2A6* and other *CYP2A* genes.

The SYBR green genotyping assays were developed using control samples of known *CYP2A6* genotype and validated by genotyping samples drawn from multiple different study populations with both the endpoint PCR assays (Method 1) and the SYBR green assays (Method 2) to assess concordance (Supplementary Table 4). The guiding principle for primer design for SYBR green PCR technology included: melting temperature of 58–60°C, primer length of approximately 20 bases, amplicon size of 50–150 base pairs, GC content of 20–80%, avoidance of runs of identical nucleotides, minimal 3′ Gs and/or Cs, and minimal probability of self- and/or hetero-dimerization (manufacturer's recommendations). To further minimize off-target amplification (in addition to using a gene-specific first amplification), allele-specific primers were designed to circumvent stretches of *CYP2A6* sequence that are 100% homologous to either *CYP2A7* or *CYP2A13*. Non-mismatch and mismatch primers were both tested and the primer pair yielding the greatest allelic discrimination was chosen [46, 47].

2.1. Protocols for *CYP2A6* SNPs & small indels

Two separate first amplifications that cover the gene from 5′ of *CYP2A6* (-1417) to intron 5 (3638) and from intron 6 (4892) to 3′ of *CYP2A6* (8548), referred to as the 5′ and 3′ region templates, respectively (Figure 1B), are utilized as a template for all SNP and indel allele-specific assays to reduce the DNA required for comprehensive *CYP2A6* genotyping and to increase throughput (primers Table 3, conditions Table 4A). The 5′ and 3′ region templates were developed using a regular thermocycler. Allele-specific SYBR green assays for the *CYP2A6*9, *20, *23, *24* and **31* alleles utilize the amplified 5′ region template while allele-specific SYBR green assays for the *CYP2A6*17* and **35* alleles utilize the amplified 3′ region template (primers Table 3, conditions Table 4B).

The SYBR green allele-specific second amplifications for the different *CYP2A6* alleles utilize similar cycling conditions and reagent concentrations (Table 4B), which enables multiple assays to be run on the same plate. Importantly, the 5′ and 3′ region templates require dilution for use as the template in the allele-specific SYBR green assays. Serial dilutions (in PCR-grade water) from 10X to 1,000X were assayed and the lowest dilution that offered optimal discrimination was chosen (10X). Undiluted first amplifications can be stored at −30°C. Anecdotally, stored first amplification templates have been used for over a year after freezing with no change in assay performance. In addition to 94 well plates, the assays could be re-optimized for 384 well plates, which would require smaller reaction volumes and reagent quantities. As guidance for genotyping calls, typical delta Ct values for each genotype (from subtracting amplification in the 'variant' well from the 'wild-type' well) are as follows: >-10 for wild-type/wildtype, −2 to +2 for wild-type/variant, and >10 for variant/variant.

2.2. Protocols for *CYP2A6* structural variants

The *CYP2A6*1B* SYBR green allele-specific second amplification uses the 3′ region template described in section 2.1 as template (Table 3 for primers and Table 4A for conditions) and targets the *CYP2A7* gene conversion the 3′ untranslated region of *CYP2A6* (Table 3 for primers, Table 4B for conditions). First and second amplification primers for the *CYP2A6*12* and **34* hybrid allele real time genotyping assays are presented (Table 3).

Reaction mixtures, cycling and gel electrophoresis conditions are also provided (Table 4A for first amplifications, Table 4B for second amplifications). The common first amplification for the *CYP2A6*12* and **34* alleles is a modified version of the 5′ region first amplification (Method 2.1). The *CYP2A6*12* and **34* alleles are a hybrid of *CYP2A6* and *CYP2A7* consisting of *CYP2A7* from 5′ through to introns 2 and 4, respectively. As such the 5′ region first amplification forward primer (Method 2.1) was re-designed to detect a region of *CYP2A6-CYP2A7* sequence homology.

Detection of the *CYP2A6*4A-H* deletion alleles by real time with SYBR green technology would require individual assays designed around the breakpoints for each deletion allele, whereas a single endpoint PCR genotyping assay (described above) can detect most of the **4* deletion alleles (Method 1.2). Other real time based PCR approaches have been developed to non-specifically detect *CYP2A6* whole deletion alleles [48, 49]. Furthermore, in future, approaches similar to those used by *Gaedigk et al.* to simultaneously detect *CYP2D6* hybrid and duplication alleles could be developed for *CYP2A6*, such as multiplex long-range PCR [50] and quantitative multiplex PCR amplification [51].

2.3 Using first amplifications as template in Method 1

The 5′ and 3′ region gene specific (first) amplifications presented in Method 2.1 can be utilized as template in the endpoint variant allele specific (second) amplifications in Method 1.1. As a guide, modifications to the endpoint PCR allele-specific amplifications (Method 1.2) include adjustments to the amount of first amplification template (0.6–0.8 uL), an increase in primer concentrations (200–500 nM), a reduction in magnesium chloride (0.75 mM), adjustments to the annealing temperature and time (~20–30 seconds), and the amount of Taq DNA polymerase (~0.3 units per reaction). For the *CYP2A6*9* assay, the authors had success modifying the type of Taq DNA polymerase ($MgCl_2$ instead of $(NH_4)_2SO_4$).

CYP2A6 genotyping project considerations

The genotyping protocols provided may require optimization within each laboratory, as fluctuations in DNA quality and concentration, reagent concentrations, and thermocycling efficiency can have a substantial impact on PCR amplification (refer to reagent manufacturer's recommendations on optimization). Genotyping assay performance is also a function of upstream sample processing. The source of DNA (e.g. blood, saliva), DNA isolation method, and storage (including freeze/thaw) can all have an impact on the quality of the DNA. The choice of *CYP2A6* alleles to genotype depends largely on the allele frequencies within the population under study [4, 16, 17, 26]. For statistical analyses individuals are often separated by genotype into predicted reduced or normal CYP2A6 metabolic activity groups [5, 6] with the caveat that activity predictions may be substrate specific. Gene variants affecting the amount of CYP2A6 protein (e.g. *CYP2A6*4* deletion allele) are expected to have a similar impact on all substrates; whereas, gene variants that influence enzymatic affinity and catalytic activity may differentially affect the metabolism of each substrate. For instance, both the *CYP2A6*17* and *CYP2A6*35* alleles, which are characterized by SNPs resulting in amino acid substitutions, appear to have a limited effect on coumarin metabolism but confer a substantial reduction in nicotine metabolism [35, 52].

Future perspective

The PCR genotyping methods outlined offer the ability to perform comprehensive *CYP2A6* genotyping with minimal genomic DNA requirements and can be adjusted at minimal cost as novel variants are discovered. These assays along with commercial TaqMan assays will likely remain the main genotyping methods in the near-term. Higher throughput methods for the simultaneous detection of *CYP2A6* copy number and hybrid variants similar to the quantitative multiplex PCR amplification recently developed for *CYP2D6* [51] would also be of benefit near-term. Genome wide methods using tag SNPs or sequencing data to investigate the association of genetic variation with drug metabolism and outcomes, while increasingly utilized, appear to have limited utility for genes with complex architecture (i.e. insertion/deletions, copy number variation) and a high degree of homology with neighboring genes, such as *CYP2A6*, *CYP2D6* and other pharmacogenes [21, 22, 53]. In the longer term, with improvements in the accuracy, cost and bioinformatics of sequencing (e.g. computational tools to resolve genotypes [54]), it may be possible to genotype *CYP2A6* using sequence data. In the meantime, real time PCR approaches are likely to continue to be utilized for *CYP2A6* genotyping, and genotyping is likely to be expanded to include additional regulatory factors, such as miR-126* sites and possibly copy number information for the *CYP2A7* pseudogene, which could be influencing *CYP2A6* transcript levels [55].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Executive Summary

Background

- Genetic variation in *CYP2A6* results in altered metabolism of CYP2A6 substrates, such as nicotine and the anti-cancer agents, letrozole and tegafur, and *CYP2A6* genotype is associated with cigarette smoking behaviors and the risk of tobacco-related cancers
- *CYP2A6* polymorphisms include SNPs, small deletions/insertions, gene deletions and duplications, and gene hybrids and conversions
- The prevalence of individual alleles varies greatly across world populations contributing to population differences in CYP2A6 activity

Genotyping strategy

- The challenge of *CYP2A6* genotyping lies predominantly in the high degree of sequence homology between *CYP2A6* and neighboring *CYP2A* genes, and to the presence of many structural variants typically requiring more labor-intensive methods to ensure accurate genotype calls
- The general genotyping strategy is a nested PCR approach whereby the first amplification, which is gene-specific, serves as a template for an allele-specific second amplification, typically designed around a 'defining/functional' SNP or indel within each allele or the *CYP2A6-CYP2A7* crossover region in the case of structural variants

Method 1

- Endpoint PCR assays using traditional thermocycling and gel electrophoresis are presented for the SNPs/indels, *CYP2A6*2, *7, *8, *9, *10, *17, *20, *23-*28, *31, *35*, and for the structural variants, *CYP2A6*1B, *1X2A*, **1X2B, *4, *12,* and **34*

Method 2

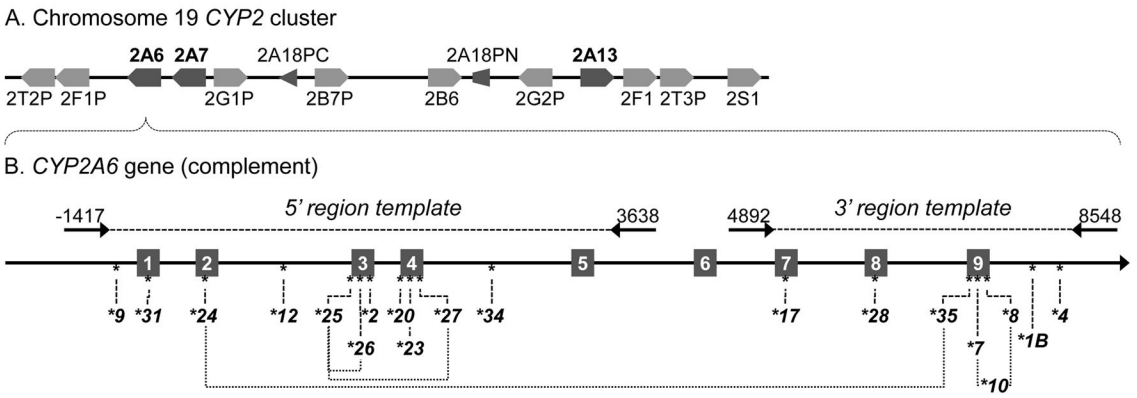
- Real time SYBR green PCR assays are presented for the SNPs/indels, *CYP2A6*9, *17, *20, *23, *24*, **31* and **35*, and for the structural variants, *CYP2A6*1B, *12* and **34*
- The real time platform offers immediate time-savings over traditional endpoint PCR by eliminating the need for gel electrophoresis to visualize results, and the use of SYBR green to detect amplification has the advantage of ease and low cost of assay modification

Genotyping project considerations

- The PCR genotyping methods outlined offer the ability to perform comprehensive *CYP2A6* genotyping with minimal DNA requirements and can be adjusted at minimal cost as novel variants are discovered

Future Perspective

- Until the technical and bioinformatics challenges of genome wide approaches are solved, variation in *CYP2A6* is best interrogated with pharmacogenetic versus genomic approaches and will increasingly rely on higher throughput real time based PCR methods



C. Description of *CYP2A6* variants

9	-48T>G	rs28399433	***2***	1799 T>A (L160H)	rs1801272	***28***	5750 G>C (E419D)	rs8192730
31	16 A>C (M6L)	rs72549432	***20***	2141_2142 del AA)		***35***	6458 A>T (N438Y)	rs61736436
24	594 G>C (V110L)	rs72549435 & ***35***	***23***	2161 C>T (R203C)	rs56256500	***7***	6558 T>C (I471T)	rs5031016
12	hybrid exon 1-2 *CYP2A7*		***27***	2162_2163 CG>A	rs28399445 & ***25***	***8***	6600 G>T (R485L)	rs28399468
25	1672 T>C (F118L)	rs28399440	***34***	hybrid exons 1-4 *CYP2A7*		***4***	deletion, assay detects *4A, D-H*	
26	1711 T>G (S131A)	rs59552350 & ***25***	***17***	5065 G>A (V365M)	rs28399454	***1B***	*CYP2A7* gene conversion in 3' UTR	

Figure 1.

Location of *CYP2A6* gene and commonly genotyped variants. **A**. *CYP2A6* within the *CYP2* cluster of genes on chromosome 19 (modified from Hoffman and Hu, 2007). **B**. *CYP2A6* gene indicating exons 1–9 (numbered boxes), commonly genotyped variants (* alleles) and gene-specific 5′ and 3′ region amplicons utilized as template in allele-specific SYBR green assays (Methods 2.1). The *CYP2A6*12*, **34* genotyping assays use a modified version of the 5′ region amplicon (Method 2.2). *CYP2A6-CYP2A7* crossover positions are illustrated for the hybrid/deletion alleles, *CYP2A6*12*, **34*, **4* and **1B*. **C**. Description of commonly genotyped *CYP2A6* variants including variation in reference gene template and amino acid sequence (if applicable).

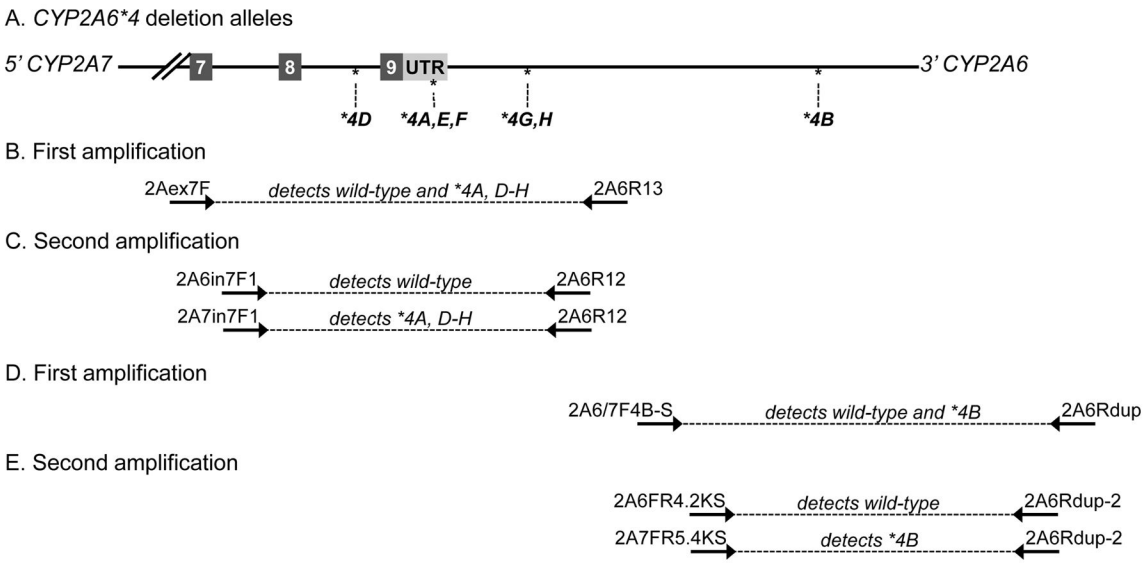


Figure 2.

*CYP2A6*4* as an illustration of a *CYP2A6-CYP2A7* hybrid allele. **A**. Crossover positions of the *CYP2A6*4* alleles indicated relative to exons (numbered boxes). **B**. First amplification of PCR genotyping of *CYP2A6*4H* detects either wild-type *CYP2A6* or all of the deletion alleles (except for **4B* which is further downstream) using a *5′* primer that anneals to either *CYP2A6* or *CYP2A7*, 2Aex7F, and a *3′* primer specific for *CYP2A6*, 2A6R13. **C**. Second amplification distinguishes between the wild-type allele and the deletion alleles using a *5′* primer that is specific to *CYP2A6*, 2A6in7F1, or to *CYP2A7*, 2A7in7F1, and a *3′* primer specific to *CYP2A6*, 2A6R12. **D**. First amplification of PCR genotyping of *CYP2A6*4B* detects either wild-type *CYP2A6* or the **4B* deletion allele using a *5′* primer that anneals to either *CYP2A6* or *CYP2A7*, 2A6/7F4B-S, and a *3′* primer specific for *CYP2A6*, 2A6Rdup. **E**. Second amplification distinguishes between the wild-type allele and the **4B* deletion alleles using a *5′* primer that is specific to *CYP2A6*, 2A6FR4.2KS, or to *CYP2A7*, 2A7FR5.4KS, and a *3′* primer specific to *CYP2A6*, 2A6Rdup-2. UTR: untranslated region

Table 1

Primer combinations for *CYP2A6* endpoint PCR genotyping (Method 1.1 & 1.2)

Genotying Assay	Forward primer	Reverse primer	Product size (bp)	Start position[b]	End position[b]
First amplifications					
*CYP2A6*9, *31*	2A65Pr1F	2A6in1R	1741	−1417	324
*CYP2A6*2, *24, *25, *26*	2A61F	2A61R	2055	144	2199
*CYP2A6*20, *23, *27*	2A6exin3F	2A6in5R	1834	1804	3638
*CYP2A6*12, *34*	2AinF-L	2A6in5R	3322	316	3638
*CYP2A6*1B, *17, *28, *35*	2A6in6F1	2A6R13	3656	4892	8548
*CYP2A6*4H*	2Aex7F	2A6R13	3538	5010	8548
*CYP2A6*4B*	2A6/7F4B-S	2A6Rdup	3098	10679	13776
*CYP2A6*7, *8, *10*	2A6in6F1	2A6R6	3127	4892	8019
*CYP2A6*1X2A*	2Aex7F	2A7R11	*a*	5010	8800
*CYP2A6*1X2B*	2A6F3	2A6/7R	6745	7076	13821
Second amplifications					
*CYP2A6*9*	2A6-460F	2A6-17RA	441	−466	−26
	2A6-460F	2A6-17RC	441	−466	−26
*CYP2A6*31*	2A6-460F	2A6ex1Rw	502	−466	36
	2A6-460F	2A6ex1Rv	502	−466	36
*CYP2A6*2*	2A62wtF	E3R-1	96	1786	1882
	2A62v1F	E3R-1	96	1786	1882
*CYP2A6*24*	2Aex2Fwt	E3R-1	1303	579	1882
	2Aex2Fv	E3R-1	1303	579	1882
*CYP2A6*25*	2A6in2ex3Fw	E3R-1	229	1653	1882
	2A6in2ex3Fv	E3R-1	229	1653	1882
*CYP2A6*26*	2A6ex2Fwt	2A6ex3R1711w	1147	579	1726
	2A6ex2Fwt	2A6ex3R1711v	1147	579	1726
*CYP2A6*20*	2A6in3F	2A6ex42144Rw	189	1969	2158
	2A6in3F	2A6ex42144Rv	189	1969	2158
*CYP2A6*23*	2A6ex42161Fw-M	5M13FOR-H2	121	2139	2260
	2A6ex42161Fv-M	5M13FOR-H2	121	2139	2260
*CYP2A6*27*	2A6in3F	2A6*4171w-M	213	1969	2182
	2A6in3F	2A6*4171v-M	213	1969	2182
*CYP2A6*12*	2A6in/ex2	2A6inex5R	3123	436	3559
	2A7in/ex2	2A6inex5R	*a*	954	3559
*CYP2A6*34*	2A6in4F	2A6inex5R	986	2573	3559
	2A7in4F	2A6inex5R	*a*	2977	3559
*CYP2A6*1B*	2A6*1Bwt	2A6R12	1559	6719	8278
	2A6*1Bvar	2A6R12	*a*	7110	8278

Genotying Assay	Forward primer	Reverse primer	Product size (bp)	Start position[b]	End position[b]
*CYP2A6*17*	2A6*17Fwt-M	2A6in7AS	382	5044	5426
	2A6*17Fv-M	2A6in7AS	382	5044	5426
*CYP2A6*28*	2A6in7F1	2A6ex8R2wt	569	5200	5769
	2A6in7F1	2A6ex8R2v	569	5200	5769
*CYP2A6*35*	2A6in8ex9F6458w	2A6R12	1835	6443	8278
	2A6in8ex9F6458v	2A6R12	1835	6443	8278
*CYP2A6*4H*	2A6in7F1	2A6R12	3078	5200	8278
	2A7in7F1	2A6R12	*a*	5594	8278
*CYP2A6*4B*	2A6FR4.2KS	2A6Rdup-2	2446	10770	13216
	2A7FR5.4KS	2A6Rdup-2	*a*	12450	13216
*CYP2A6*7*	2A6*7Fwt-M	2A6R0	1244	6539	7783
	2A6*7Fv-M	2A6R0	1244	6539	7783
*CYP2A6*8*	2A6*8wtF	2A6R0	1201	6582	7783
	2A6*8vF	2A6R0	1201	6582	7783
*CYP2A6*10*	2A6*7Fwt-M	2A6*8Rwt-L	81	6539	6620
	2A6*7Fwt-M	2A6*8Rv-L	81	6539	6620
	2A6*7Fv-M	2A6*8Rwt-L	81	6539	6620
	2A6*7Fv-M	2A6*8Rv-L	81	6539	6620
*CYP2A6*1x2A*	2A7in7F1	2A7R12	3093	5594	8687
	2A6in7F1	2A7R12	*a*	5200	8687
*CYP2A6*1x2B*	2A6F0	2A6Rdup	6041	7735	13776
	2A6F0	2A7Rdup	*a*	7735	15131

[a] Multiple slightly different product sizes are possible depending on where the crossover junctions are located. The differences in size between the hybrid alleles were not resolved on our 1.2% agarose gels

[b] *CYP2A6* primer position is based on *CYP2A6* +1ATG, and *CYP2A7* primer position is based on *CYP2A7* +1ATG

Table 2A

Polymerase chain reaction first-step amplification conditions (Method 1.1 & 1.2)

	First-step amplification									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Buffer	2.5[a]	2.5[b]	2.5[a]	2.5[a]	2.5[a]	2.5[c]	2.5[a]	2.5[a]	2.5[b]	2.5[d]
DNA (ng)	50	50	50	50	50	50	50	50	50	75
Primers (each) (nM)	62.5	125	62.5	125	125	100	125	125	62.5	250
DMSO	–	–	–	–	–	–	–	–	–	2%
dNTPs (uM)	200	200	200	200	200	125	200	200	200	300
$MgCl_2$ (mM)	1.3	2.0	1.5	1.5	1.5	–	2.5	1.5	1.7	–
Taq (units)	0.75[ɨ]	0.75[ɨ]	1.25[ɨ]	0.75[ɨ]	1.00[ɨ]	0.5uL[£]	1.25[ɨ]	1.00[ɨ]	1.25[ɨ]	1.25[₮]
First denaturation (°C:sec)	95:60	95:60	95:60	95:60	95:60	95:120	95:60	95:60	95:60	94:30
[i]Denaturation (°C:sec)	95:20	95:15	95:15	95:15	95:15	95:20	95:15	95:15	95:15	94:20
[i]Annealing(°C:sec)	55:30	60:30	58:30	60:30	50:30	50:20	52:20	50:30	53:30	54:20
[i]Extension(°C:sec)	72:60	72:120	72:120	72:210	72:210	72:60	72:180	72:180	72:180	68:420
[ii]Denaturation (°C:sec)	–	–	–	–	–	–	–	–	–	94:20
[ii]Annealing(°C:sec)	–	–	–	–	–	–	–	–	–	54:20
[ii]Extension(°C:sec)	–	–	–	–	–	–	–	–	–	68:420[e]
Number of cycles	35	36	30	35	40	30	35	40	35	_[g]
Last extension(°C:min)	72:7	72:7	72:7	72:7	72:7	72:3	72:7	72:7	72:7	68:10

1: First amplification for *CYP2A6*9, *31*

2: First amplification fo *CYP2A6*2, *24, *25, *26*

3: First amplification fo *CYP2A6*20, *23, *27*

4: First amplification fo *CYP2A6*12, *34*

5: First amplification fo *CYP2A6*1B, *17, *28, *35*

6: First amplification fo *CYP2A6*4H*

7: First amplification fo *CYP2A6*4B*

8: First amplification fo *CYP2A6*7,*8,*10*

9: First amplification fo *CYP2A6*1X2A*

10: First amplification fo *CYP2A6*1X2B*

[ɨ] Taq DNA Polymerase (Thermo Scientific)

[£] Pfu: PfuUltra II Fusion HS DNA Polymerase (Agilent Technologies)

[₮] Long PCR Enzyme Mix (Fermentas, Canada)

[a] 10X Taq Buffer with $MgCl_2$ (Thermo, Canada)

[b] 10X Taq Buffer with $(NH_4)_2SO_4$ (Thermo, Canada)

[c] 10X Pfu Buffer (Agilent Technologies)

[d] 10X Long PCR Buffer with $MgCl_2$ (Fermentas, Canada)

[e] +5 seconds per cycle

[g] Ten cycles for [i], 20cycles for [ii]

Table 2B

Polymerase chain reaction second-step amplification conditions (Method 1.1 & 1.2)

	Second-step amplification																					
	CYP2A6*9	***CYP2A6*31***	***CYP2A6*2***	***CYP2A6*24***	***CYP2A6*25***	***CYP2A6*26***	***CYP2A6*20***	***CYP2A6*23***	***CYP2A6*27***	***CYP2A6*12***	***CYP2A6*34***	***CYP2A6*1B***	***CYP2A6*17***	***CYP2A6*28***	***CYP2A6*35***	***CYP2A6*4H***	***CYP2A6*4B***	***CYP2A6*7***	***CYP2A6*8***	***CYP2A6*10***	***CYP2A6*1x2A***	***CYP2A6*1x2B***
Buffer	2.5[b]	2.5[b]	2.5[a]	2.5[a]	2.5[a]	2.5[a]	2.5[a]	2.5[a]	2.5[a]	2.5[a]	2.5[a]	2.5[b]	2.5[b]	2.5[a]	2.5[a]	2.5[b]	2.5[a]	2.5[a]	2.5[b]	2.5[b]	2.5[b]	2.5[c]
First-step product (µL)	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Primers (each) (nM)	62.5	62.5	125	75	100	62.5	125	150	125	125	62.5	125	250	125	125	125	125	250	150	100	125	250
DMSO	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2%
dNTPs (µM)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	300
$MgCl_2$ (mM)	1.10	1.10	1.25	1.25	0.75	1.20	1.50	1.00	1.30	1.50	1.50	1.50	1.00	1.50	1.25	1.50	1.50	1.10	1.20	1.30	1.60	–
Taq (units)	0.4[Ŧ]	0.4[Ŧ]	0.25[Ŧ]	0.3[Ŧ]	0.3[Ŧ]	0.4[Ŧ]	0.3[Ŧ]	0.4[Ŧ]	0.4[Ŧ]	0.5[Ŧ]	0.5[Ŧ]	0.5[Ŧ]	0.5[Ŧ]	0.3[Ŧ]	0.5[Ŧ]	0.5[Ŧ]	0.5[Ŧ]	0.3[Ŧ]	0.4[Ŧ]	0.3[Ŧ]	0.75[Ŧ]	1.25[₮]
First denaturation (°C:sec)	95:60	95:60	95:60	95:60	95:60	95:60	95:60	95:60	95:60	95:60	95:60	95:60	95:60	95:60	95:60	95:60	95:60	95:60	95:60	95:60	95:60	94:30:00
[i]Denaturation (°C:sec)	95:20	95:20	95:15	95:15	95:15	95:15	95:15	95:15	95:15	95:15	95:15	95:15	95:15	95:15	95:15	95:15	95:15	95:15	95:15	95:15	95:15	94:20
[i]Annealing(°C:sec)	66:40	65:30	50:20	58:10	65:10	59:20	56:20	62:10	56:20	62:20	59:20	52:30	58:30	58:20	55:40	52:30	50:20	59:20	57:20	57:30	60:30	54:20
[i]Extension(°C:sec)	72:60	72:60	72:45	72:90	72:45	72:60	72:30	72:30	72:40	72:120	72:60	72:120	72:60	72:60	72:60	72:180	72:90	72:60	72:60	72:30	72:150	68:420
[ii]Denaturation (°C:sec)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	94:20
[ii]Annealing(°C:sec)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	54:20
[ii]Extension(°C:sec)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	68:420[d]
Number of cycles	20	20	22	24	16	18	18	23	18	25	22	20	25	20	20	25	20	30	20	30	25	–[e]
Last extension(°C:min)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	68:10	

[Ŧ] Taq DNA Polymerase (Thermo Scientific)

[₮] Long PCR Enzyme Mix (Fermentas, Canada)

[a] 10X Taq Buffer with $MgCl_2$ (Thermo, Canada)

[b] 10X Taq Buffer with $(NH_4)_2SO_4$ (Thermo, Canada)

[c] 10X Long PCR Buffer with $MgCl_2$ (Fermentas, Canada)

[d] +5 seconds per cycle

[e] Ten cycles for [i], 20cycles for [ii]

Table 3

Primers combinationsfor *CYP2A6* genotyping real-time PCR genotyping (Method 2.1 and 2.2)

Assay	Primer Name	Location[a]	Direction	Sequence
First amplifications				
5′ region template:	2A65Pr1F	5′ flanking (−1417)	F	ACC TAG ACT TAA TCT TCC CGT ATA C
*CYP2A6*9, *31, *24, *20, *23*	2A6in5R	Intron 5 (3638)	R	GGC CTG TGT CAT CTG CCT
Modified 5′ region:	2Ain1F-L	Intron 1 (316)	F	GAT CTT GGG ATG TCC AGC TCC
*CYP2A6*12, *34*	2A6in5R	Intron 5 (3638)	R	GGC CTG TGT CAT CTG CCT
3′ region template:	2A6in6F1	Intron 6 (4892)	F	ATTTCCTGCTCTGAGACC
*CYP2A6*17, *35, *1B*	2A6R13	3′ flanking (8548)	R	GCC TCC CAT AGT GCT ATA ATT AAC A
Second amplifications				
*CYP2A6*9*	2A6*9wtR-Sybr-M[b]	5′ flanking (−29)	R	GCT GGG GTG GTT TGC CTC TA
	2A6*9vR-Sybr-M[b]	5′ flanking (−29)	R	GCT GGG GTG GTT TGC CTC TC
	125M13BEV-B	5′ flanking (−146)	F	CCC AAG CTA GGC AGG ATT CAT G
*CYP2A6*31*	2A6*31wtR-Sybr	Exon 1 (35)	R	AGC AAG GCC ACC AGA AGC AT
	2A6*31vR-Sybr	Exon 1 (35)	R	AGC AAG GCC ACC AGA AGC AG
	2A61F-L	Exon 1 (−155)	F	TGG CTG TGT CCC AAG CTA GGC A
*CYP2A6*24*	2A6*24wtR-Sybr-M[b]	ExIn 2 (619)	R	ACC CCC TCA CCA TAG CCT TTG AAT AC
	2A6*24vR-Sybr-M[b]	ExIn 2 (619)	R	ACC CCC TCA CCA TAG CCT TTG AAT AG
	2A6ex2-505F	Exon 2 (505)	F	TGC TGT GTG GAC ATG ATG CCG TCA G
*CYP2A6*20*	2A6*20wtR-sybr-M[b]	Exon 4 (2160)	R	CAA CAG TGA CAG GAA CTC TTT GTA CT
	2A6*20vtR-sybr-M[b]	Exon 4 (2160)	R	CAA CAG TGA CAG GAA CTC TGT ACT
	2A6in3F-L	Intron 3 (1967)	F	CCC TGC CTC CTG GAA TTC TGA C
*CYP2A6*23*	2A6ex42161Fw-M[b]	Exon 4 (2139)	F	CAA AGA GTT CCT GTC ACT GTC GC
	2A6ex42161Fv-M-L[b]	Exon 4 (2137)	F	GA CAA AGA GTT CCT GTC ACT GTC GT
	5M13FOR-H2	Intron 4 (2260)	R	GCA GTT GGC AGG TTG TGG TAG G
*CYP2A6*12*	2A6in1/ex2-L	In1/Ex2 (434)	F	CCACCTCCATCAGATCAGTGAGC
	2A7in1/ex2-L	In1/Ex2 (952)	F	TCGCCTCCATCAGTTCAGTGAGT
	2A6R-667	Intron 2 (667)	R	GAACACTGAGACCTTCGTGTCCA
*CYP2A6*34*	2A6-2572F	Intron 4 (2572)	F	TCAACCGGCCTCCTGCATA
	2A7-2978F	Intron 4 (2978)	F	AAC CCG CCT CCT GCA TG
	2A6-2862R	Intron 4 (2862)	R	GTTTACCTATCCAAGTGGGATGCACT
*CYP2A6*17*	2A6*17Fwt-Sybr-M[b]	Exon 7 (5042)	F	ACG AGA TCC AAA GAT TTG GAG CCG
	2A6*17Fv-Sybr-M[b]	Exon 7 (5042)	F	ACG AGA TCC AAA GAT TTG GAG CCA
	2A6in7-17R2	Intron 7 (5198)	R	GGA TGC TGG GGA CAC AGA GAG

Assay	Primer Name	Location[a]	Direction	Sequence
*CYP2A6*35*	2A6*35wtR-Sybr	Exon 9 (6479)	R	CCA GGC CTT CTC CGA AAC AGT T
	2A6*35vR-Sybr	Exon 9 (6479)	R	CCA GGC CTT CTC CGA AAC AGT A
	2A6in8-6395F	Intron 8 (6395)	F	CGA GGC TGC ACT GAG AGT GG
*CYP2A6*1B*	2A6*1Bwt	3′ flanking (6719)	F	ACT GGG GGC AGG ATG GC
	2A6*1Bvar-L	3′ flanking (7101)	F	GGG TAT AAG AAT GGG GGG AAG ATG CG
	2A6R6944	3′ flanking (6944)	R	GTG GCA ATT AGG TGA GCG TGC AAT G

[a] *CYP2A6* primer position is based on *CYP2A6* +1ATG, and *CYP2A7* primer position is based on *CYP2A7* +1ATG

[b] Mismatch primer at third position from 3′ end, underlined in sequence

Table 4A

Polymerase chain reaction first-step amplification conditions (Methods 2.1 & 2.2)

	First-step amplification		
	I	**II**	***12, *34***
Buffer	1xPfu buffer	1xPfu buffer	1X Taq Buffer with MgCl2
DNA (ng)	50	50	50
Primers (each) (nM)	100	100	125
dNTPs (uM)	62.5	62.5	200
$MgCl_2$ (mM)	na	na	1.5
Taq[Ɨ]	0.5uL to 25ul[Ɨ]	0.5uL to 25ul[Ɨ]	0.75U to 25ul [£]
First denaturation (°C:sec)	95:120	95:120	95:60
Denaturation (°C:sec)	95:20	95:20	95:15
Annealing(°C:sec)	60:15	55:30	60:30
Extension(°C:sec)	72:75	72:60	72:210
Number of cycles	40	40	35
Last extension(°C:sec)	72:180	72:180	72:7

I: First amplification for 1st region (*CYP2A6*9, *20, *23, *24, *31*)

II: First amplification for 2nd region (*CYP2A6*17, *35, *1B*)

[Ɨ]Pfu: PfuUltra II Fusion HS DNA Polymerase (Agilent Technologies)

[£]Taq DNA Polymerase (Thermo Scientific)

Table 4B

SYBR Green Polymerase chain reaction second-step amplification conditions (Methods 2.1 & 2.2)

	Second-step alleles (96 well)[Ł]		
	20, *23, *24, *31, *1B	***9, *17, *35***	***12, *34***
First -step prodoct (uL)[£]	1	1	1
Primers (each) (nM)	125	125	125
SYBRGreen supermix (2x)[₮]	5uL to 10uL	5uL to 10uL	5uL to 10uL
Hold stage (°C:min)	95:10	95:10	95:10
PCR Stage			
Denaturation (°C:sec)	95:15	95:15	95:15
Annealing(°C:sec)	65:5	65:10	65:10
Number of cycles	50	50	50
Melt Curve stage			
Denaturation (°C:sec)	95:15	95:15	95:15
Annealing(°C:sec)	60:60	60:60	60:60

[Ł] ViiA 7 Real Time PCR System (Applied Biosystems)

[£] Diluted 10 times

[₮] iTaqTM Universal SYBRGreen Supermix (Bio-Rad)