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High-resolution melt analysis to detect sequence variations in highly homologous gene regions: application to *CYP2B6*

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Abstract

High-resolution melt (HRM) analysis using 'release-on-demand' dyes, such as EvaGreen[®] has the potential to resolve complex genotypes in situations where genotype interpretation is complicated by the presence of pseudogenes or allelic variants in close proximity to the locus of interest. We explored the utility of HRM to genotype a SNP (785A>G, K262R, rs2279343) that is located within exon 5 of the *CYP2B6* gene, which contributes to the metabolism of a number of clinically used drugs. Testing of 785A>G is challenging, but crucial for accurate genotype determination. This SNP is part of multiple known *CYP2B6* haplotypes and located in a region that is identical to *CYP2B7*, a nonfunctional pseudogene. Because small *CYP2B6*-specific PCR amplicons bracketing 785A>G cannot be generated, we simultaneously amplified both genes. A panel of 235 liver tissue DNAs and five Coriell samples were assessed. Eight *CYP2B6/CYP2B7* diplotype combinations were found and a novel variant 769G>A (D257N) was discovered. The frequency of 785G corresponded to those reported for Caucasians and African-Americans. Assay performance was confirmed by *CYP2B6* and/or *CYP2B7* sequence analysis in a subset of samples, using a preamplified *CYP2B6*-specific long-range-PCR amplicon as HRM template. Inclusion rather than exclusion of a homologous pseudogene allowed us to devise a sensitive, reliable and affordable assay to test this *CYP2B6* SNP. This assay design may be utilized to overcome the challenges and limitations of other methods. Owing to the flexibility of HRM, this assay design can easily be adapted to other gene loci of interest.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

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Keywords

CYP2B6; *CYP2B7*; genotyping; high-resolution melt analysis; HRM; SNPs

High-resolution melt (HRM) analysis is a method that detects the presence of sequence variations in real-time after the completion of a PCR reaction [1]. This method has become substantially more sensitive with the development of so-called 'release-on-demand dyes', such as EvaGreen®, in concert with the improvement of instrumentation to measure the dissociation, or 'melt', behavior of DNA fragments. HRM can now reliably detect one or more sequence variations including SNPs and nucleotide insertions and deletions (indels) in DNA in medium- and high-throughput formats. It presents a powerful alternative to other methods such as time-consuming and labor-intensive gel-based RFLP and allele-specific PCR assays, or assays that require fluorescence-labeled hybridization probes such as TaqMan® among others. The utility of HRM has been described for a wide range of applications, for example, viral and microbial strain differentiation [2–4], to screen for DNA sequence variations [4–7], as well as clinical testing and diagnostics [8,9], including the sensitive detection of somatic mutations in cancer [10].

Drug metabolizing enzymes, such as the members of the CYP450 superfamily, are notoriously difficult to genotype owing to high degrees of homology among closely related genes and the presence of pseudogenes. Therefore, genotyping assays demand utmost attention during development and application to ensure that only the gene of interest is amplified and that interpretation of results is not compromised by the presence of unspecific fragments, generated related genes and/or pseudogenes.

The human *CYP2B* gene locus is no exception [11,12]. This highly polymorphic gene locus comprises two genes, the nonfunctional *CYP2B7* pseudogene and the *CYP2B6* gene, which encodes a functional enzyme that metabolizes several clinically useful medications, including the dopamine reuptake inhibitor bupropion [13–15], the antiviral drugs efavirenz and nevirapine, mainstay drugs to treat HIV/AIDS [16–19], the sedative propofol [20,21], the antiaddiction drug methadone [22] and the bioactivation of cyclophosphamide [23–25], an agent used in chemotherapy and to treat various non-neoplastic autoimmune diseases [12,26,27]. As summarized by Thorn *et al.*, *CYP2B6* is a very important pharmacogene [28], however, the utilization and implementation of genetic information for making personalized treatment decisions, still requires further investigation. Of importance, there appear to be significant substrate-dependent effects regarding the activity conferred by particular *CYP2B6* allelic variants. For example, *CYP2B6**6 has been associated with decreased metabolism of bupropion and efavirenz, but this does not seem to be the case for cyclophosphamide. Hence, correct *CYP2B6* allele assignment is important for phenotype classification and appropriate genotype to phenotype interpretations and conclusions, as argued by Helsby *et al.* [29].

Currently, The Human CYP450 Allele Nomenclature Database defines 30 allelic variants for *CYP2B6* [101]. As shown in Figure 1, 785A>G (K262R) is part of multiple *CYP2B6* haplotypes. If 785A>G, or other SNPs that are in linkage disequilibrium with 785A>G, are not tested, allele assignment may not be accurate and the resulting genotype assignments may lead to inaccurate phenotype predictions [29]. Published methods to genotype 785A>G include RFLP analysis of larger amplicons and/or sequencing using *CYP2B6*-specific amplification products [13,30], a combination of multiplex PCR and allele-specific PCR [31], multiplex genotyping by MALDI-TOF [32] or, as recently described, allele-specific primer extension using the Luminex (TX, USA) xMAP platform [33]. Exon 5 and adjacent intron sequences are near-identical to the analogous region of *CYP2B7*. In addition to the

variations shown in Figure 1, there are only four potential single nucleotide differences in >500 bp upstream and none within 139 bp downstream of 785A>G. This makes gene-specific amplification of the immediate region flanking the 785A>G SNP very challenging, explaining why no TaqMan assays have been developed for 785A>G, while such assays are commercially available for numerous other *CYP2B6* SNPs. Challenges of testing this particular SNP may explain why some investigators are excluding 785A>G from genotyping in their studies.

To facilitate genotype analysis of 785A>G directly from genomic DNA for our own investigations we designed an HRM assay that simultaneously amplifies both genes, *CYP2B6* and *CYP2B7*, and takes advantage of the sensitivity of HRM to distinguish multiple haplotypes that are associated with sequence variations in the exon 5 gene region. HRM melt profiles were confirmed by sequence analysis and by using a *CYP2B6*-specific preamplified long-range (XL)-PCR template. The successful design and application that is exemplified in this report on *CYP2B6* can easily be adapted to genotype other SNPs located in highly homologous gene regions.

Methods

Samples

Human liver tissue specimens were obtained from the NIH-supported tissue programs: the Liver Tissue Cell Distribution System (LTCDS; n=86), with 34 out of 86 and 52 out of 86 from the Minnesota (MN, USA) and Pittsburg (PA, USA) collection centers, respectively); the University of Maryland Brain and Tissue Bank for Developmental Disorders (MD, USA; n = 91); and the Laboratory of Developmental Biology at the University of Washington (WA, USA; n = 19). Additional samples (n = 35) were from Xenotech, LLC (KS, USA), and four tissues were obtained from Vitron (AZ, USA). The use of these tissues was declared nonhuman subjects research by the University of Missouri-Kansas City Pediatric Health Sciences Review Board (MO, USA).

The panel comprised a total of 235 tissue samples from subjects of different ethnicities (Caucasian, n = 114; African-American, n = 54; unknown and/or other, n = 67). PCR

Five DNA samples obtained from the Coriell Institute for Medical Research (NJ, USA) served as controls; these are referred to as Coriell samples.

DNA isolation

Genomic DNA (gDNA) from 235 liver tissue samples was isolated using mini spin kits from various suppliers including the Qiagen AllPrep® DNA/RNA Mini and DNeasy® Blood and Tissue Kits (Qiagen, CA, USA) and the Illustra tissue and cells genomic Prep Mini Spin Kit (GE Healthcare, NJ, USA). gDNA quality was assessed by agarose gel electrophoresis and DNA concentration determined spectrophotometrically with a NanoDrop instrument at the time of isolation.

HRM assay

The HRM PCR primers were designed to simultaneously amplify *CYP2B6* and *CYP2B7*. This 'duplexing' approach produced 70-bp long PCR products from each gene (Figure 1). HRM was performed on an Illumina Eco Real-Time PCR System (Illumina, CA, USA) using the KAPA™ HRM FAST PCR Kit (KAPA Biosciences, MA, USA) following the manufacturer's instructions for assay conditions. HRM reactions were carried out in 6 µl reaction volumes. Final concentrations were at 0.2 µM for each primer, 1× for and the KAPA reaction mix, 2.5 mM MgCl₂ 10–20 ng gDNA (Table 1). PCR cycling included

an initial denaturation step (95°C, 3 min), which was followed by 40 cycles of 95°C, 5 s denaturing and a combined annealing and extension step at 60°C for 30 s. Subsequently, PCR products were 'melted' over a 55–95°C gradient and using preset parameters of the Eco Real-Time instrument. Each sample was run in duplicate and each plate contained known controls. Genotypes/haplotypes were assigned by comparing sample curves with control curves. Samples, for which duplicates did not produce superimposing melt curves or both duplicates deviated from known melt profiles, were repeated at least once in duplicate.

HRM analysis on preamplified *CYP2B6* XL-PCR template

To obtain HRM genotype results that *CYP2B6* variation only, XL-PCR products encompassing exons 4–9 were generated (Figure 1). Each 8 µl reaction contained 1× KAPA-Long Range HotStart Taq[®] polymerase Ready-Mix with dye (KAPA Biosciences), 5% dimethyl sulfoxide, 0.5 µM of each primer (Table 1) and 10–20 ng of gDNA. PCR cycling conditions comprised initial denaturation (95°C, 3 min), followed by 35 cycles of denaturation (94°C, 15 s), annealing (61°C, 30 s) and extension (68°C, 11 min). XL-PCR fragment production was verified by gel electrophoresis. The 10,328-bp long amplicons were diluted 500- to 2000-fold with 10 mM Tris pH 8 and subsequently served as templates for HRM as described above.

DNA sequence analysis

To sequence the *CYP2B6* and *CYP2B7* exon 5 regions, gene-specific 641-bp and 608-bp long PCR fragments, respectively, were generated using KAPA 2G[™] Fast ReadyMix (KAPA Biosciences). PCR conditions were comprised of initial denaturation (95°C, 3 min) followed by 35 cycles of denaturation (95°C, 15 s), annealing (64°C, 30 s) and extension (72°C, 40 s). Subsequently, amplicons were treated with ExoSAP-IT[®] (Affymetrix, CA, USA) as recommended and sequenced with the forward and reverse primers used for their amplification with BigDye terminator v3.1 cycle sequencing chemistry on a 3730×l DNA Analyzer (Applied Bio-systems, CA, USA). Sequences were compared with the following GenBank and ENSEMBLE database entries: *CYP2B6* (NG_000008.7 [used by the CYP450 Allele Nomenclature Committee], AC023172 and ENSG00000197408) and *CYP2B7* (GenBank AC008537 and ENSEMBLE ENSG00000256612). Sequences were aligned using Sequencher software V5.0 (Gene Codes, MI, USA).

To confirm HRM results and to interpret HRM melt profiles that did not overlap with those produced from samples with known genotypes, 65 samples were sequenced for *CYP2B6* and 35 for *CYP2B7*, respectively. All samples used as controls were also sequenced for both genes.

Haplotype analysis

PHASE (version 2.1) software was used to construct haplotypes from genotype data as described [34,35].

Results

HRM analysis on gDNA

HRM analysis was panel of sequenced samples including five from the Coriell Institute to ensure that melt profiles accurately distinguished and reflected the sequence variations contained in these samples. The *CYP2B6* and *CYP2B7* diplotypes for the Coriell samples are provided in the legend of Table 2. HRM analysis was then performed on gDNA of the 235 liver tissue specimens. Figures 2A & B show normalization and differential plots representing melt profiles of the eight *CYP2B6/2B7* diplotypes that were detected in the

panel. The melt profiles for 204 samples could be unequivocally interpreted. Diplotypes were assigned by comparison of sample profiles to those generated from the reference control samples.

Repeating the HRM assay resolved ambiguities for 15 of the 31 samples whose HRM result could initially not be interpreted with confidence. Resequencing both genes, *CYP2B6* and *CYP2B7*, confirmed the result. However, 16 samples consistently exhibited melt profiles that were subtly or dramatically different from control profiles. Sequence analysis resolved the deviating melt profiles for two of these 16 samples. The first revealed the *CYP2B6**3-defining 777C>A SNP (rs45482602) while the second sample was found to be compound heterozygous for the *CYP2B6* 769G>A and 785A>G SNPs, with one variant on each chromosome. Noteworthy, the nonsynonymous 769G>A SNP (D257N) is not part of any defined allele or haplotype according to CYP450 nomenclature [101], but has been assigned a RefSeq (rs) number in dbSNP (rs34646544).

The frequencies of the eight observed *CYP2B6*/*CYP2B7* diplotype combinations are summarized in Table 2 and inferred and observed haplotypes and their respective frequencies are shown in Table 3. Two inferred haplotypes were not observed (Table 3, haplotypes 6 and 7).

Frequencies of the *CYP2B6* 785G allele were 28.5, 34.3 and 27.6% among the Caucasian, African-American and unknown/other ethnic groups, respectively, which is within the published ranges [12,17,32]. Notably, the *CYP2B6**3 allele was discovered in an African-American tissue sample (0.93%), but not in livers from Caucasians or the unknown/other groups.

Haplotype analysis using the PHASE algorithm (Table 3) indicated that diplotype ID 5 consisting of a haplotype 2/3 combination (CGCG/TGCA) is more likely than a diplotype consisting of a haplotype 1/7 combination (CGCA/TGCG). The calculated probability that a subject has the observed ID 5 diplotype was 0.75 vs 0.25% for having a 1/7 haplotype combination; we did not observe a single individual with a diplotype that would unequivocally indicate the presence or existence of haplotype 7.

HRM analysis on preamplified XL-PCR template

As the coamplification of *CYP2B7* adds to the complexity of melt curve profiles, HRM-lysis was conducted on preamplified *CYP2B6*-specific XL-PCR products encompassing exons 4–9 to confirm our results (Figure 3). This step included 30 samples representing all observed *CYP2B6*/*CYP2B7* diplotype combinations; this subset included the Coriell controls and samples presenting with aberrant melt profiles that were inconsistent with their putative DNA sequence. The approximately 10-kb long XL-PCR product was successfully amplified in 26 of the 30 samples; the four failures were likely due to DNA degradation. Although the intensity of the XL-PCR product varied considerably among the samples, XL-PCR product was visible after gel electrophoresis. The melt profiles generated from the *CYP2B6*-specific XL-PCR template were concordant with the HRM results obtained from gDNA and sequence data for 25 of the 26 samples. The only exception was the sample heterozygous for 769G>A and 785A>G. Notably, HRM using the XL-PCR product as template yielded a melt curve that matched the curve obtained for samples homozygous for 785G (diplotype ID 1, 4 and 6, Table 3). This contrasts the unique profile when HRM was performed on gDNA or the amplicon used for sequencing. Subsequent sequence analysis of the XL-PCR product demonstrated homozygosity for 785G suggesting that the allele carrying 769G>A did not amplify. This unintentional allele dropout, however, allowed us to determine that 769A and 785G were located on opposite alleles and deduce the phase of haplotype 5 (Table 3).

Discussion

We have successfully designed, optimized and evaluated a HRM assay to reliably detect multiple sequence variations in a gene region that is near identical with a pseudogene in the immediate vicinity of the SNPs of interest. Our assay design differs from conventional designs by simultaneously amplifying the interfering gene *CYP2B7*, along with the target gene *CYP2B6*. Although the melt profiles were complex, in part due to the presence of a minor allelic *CYP2B7* variant (Figures 1 & 2), the resulting HRM melt profiles accurately distinguished samples carrying *CYP2B6* 785A/A, A/G and G/G genotypes from each other. HRM also correctly identified one sample as a carrier of the rare *CYP2B6**3 (777G>A) allele and revealed a putative novel allele carrying a D257N amino acid change that may alter *CYP2B6* function.

Because 785A>G is part of multiple *CYP2B6* haplotypes, it is necessary to test this SNP to discriminate the various *CYP2B6* allelic variants from each other. Owing to the high sequence similarity between *CYP2B6* and *CYP2B7*, detection of this SNP is challenging and has traditionally been performed by more time-consuming and labor-intensive methods [13,30,31]. More recently, this SNP has also been included in *CYP2B6* genotype panels using MALDI-TOF and Luminex xMAP [32, 33] platforms, which allow high(er) throughput genotyping, but may not be available to many investigators. Regions of high homology between related genes are also still posing challenges to next-generation sequencing platforms owing to difficulties of aligning the relatively short reads to the correct gene and not their related counterparts. Unless these challenges are solved, next-generation sequencing platforms may not be able to accurately identify and assign the *CYP2B6/7* SNPs described in this report or SNPs located within regions that are (near) identical between members of a gene family. HRM is a relatively affordable method that only requires a real-time PCR instrument with HRM capability and analysis software and no costly synthesis of fluorescent probes. There are many examples in the literature describing HRM assays that have been successfully used. Among them are assays for other drug-metabolizing enzyme genes including *CYP2C8* [36], *CYP2C19* [37], *CYP2D6* [38] and *CYP21A2*, a CYP450 involved in steroid metabolism [39].

HRM is a relatively rapid, simple, yet flexible procedure that is amenable to automation and therefore offers the potential to replace gel-based assays [1]. However, there are a number of challenges and potential pitfalls. DNA quality and quantity can impact HRM melt profiles, in particular carryover of salt from the DNA isolation procedure into the HRM reaction causing low sensitivity, poor reproducibility and/or incorrect genotype calls. A small subset of 14 samples indeed produced melt profiles that were consistently distinct from all other samples even though sequence analysis suggested otherwise; this may be explained by the presence of salt or other impurities. Since the gDNAs of our liver tissue panel were isolated for multiple different projects over a sustained period of time using different kit suppliers, we speculate that some variability among the DNA preparations contributed to these inconsistent results [8,40]. Among those were DNAs compromised by various degrees of degradation (due to tissue quality), or may have contained other agents that interfered with PCR amplification and/or melt characteristics of the amplification products. We have also observed more variability in a quantitative multiplex copy number variation assay that is sensitive to DNA impurities and quality when comparing tissue DNA samples with those isolated from blood [41]. This HRM assay was also performed on gDNA extracted from blood of 37 patients (results not shown) without encountering any miscalls suggesting that uniform DNA quality across samples increase call rates and minimize false or no-calls.

Also, especially when interrogating multiple SNPs at the same time, melt patterns of different heterozygotes may be quite similar and thereby difficult or impossible to

discriminate[1,5]. All diplotypes (Table 2) in our panel revealed distinct melt profiles when the assay was performed on gDNA. However, assay validation is of utmost importance because rare and/or unknown SNPs may interfere with an assay and/or its interpretation. This is exemplified by the case exhibiting heterozygosity at positions 785 and 769, which produced different melt curves for HRM performed on gDNA and *CYP2B6*-specific XL-PCR product. This discordance may be explained, however, by the apparent allele dropout that occurs during the XL-PCR amplification. One or more of the following SNPs map within the binding site of the reverse XL-PCR primer (rs3211375, rs3211376, rs3211377 and rs3211378). None of these SNPs have been reported by dbSNP to be present in any population and it remains unclear whether these indeed interfered with XL-PCR amplification. We do not have any evidence, that this phenomenon is an issue with any other allelic variants, but this observation is a reminder that rare sequence variations do exist and can interfere with genotype analyses.

Conclusion

We demonstrated using *CYP2B6* as an example that HRM analysis is sufficiently powerful and sensitive to genotype one or multiple SNPs located in highly homologous gene regions by simultaneously amplifying the gene of interest and its homologous counterpart. This approach allowed us to successfully overcome the challenges and need to produce a single gene-specific amplification product for genotype analysis. Owing to the versatility and flexibility, this design can easily adapted to other gene loci of interest.

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Executive summary

Aim

- To assess the utility of high-resolution melt (HRM) analysis for accurate detection of SNPs in highly homologous gene regions.
- To demonstrate using the example of *CYP2B6* 785A>G, a SNP located in a region that is near identical to the *CYP2B7* pseudogene, that HRM is sufficiently sensitive to accurately detect sequence variations in this gene region.

Methods

- *CYP2B6* and *CYP2B7* were simultaneously amplified and the resulting 70-bp long amplicons subjected to HRM analysis.
- Results were confirmed by *CYP2B6*- and *CYP2B7*-specific sequence analysis and HRM analysis on a *CYP2B6*-specific long-range PCR amplicon.

Results

- HRM revealed eight distinct melt curve patterns corresponding to eight different *CYP2B6/7* diplotypes.
- The majority of samples were accurately identified by HRM; DNA quality likely contributed to melt curve patterns that could not unequivocally be interpreted.
- The rare *CYP2B6**3-defining 777C>A SNP that is located in close proximity to 785A>G was reliably revealed by HRM.
- HRM also discovered a nonsynonymous SNP (769G>A, D257N) that was not in linkage disequilibrium with any other SNP detected on the 70-bp long amplicon; 769G>A likely constitutes a novel *CYP2B6* haplotype that warrants complete characterization.

Conclusion

- HRM is a relatively affordable, yet powerful method to accurately detect multiple sequence variations and diplotypes in the *CYP2B6/7* exon 5 gene region.
- In cases where gene-specific amplifications is difficult or impossible, coamplification of two (or possibly more genes) followed by HRM analysis may be a viable approach for SNP detection and/or discovery.

Figure 1. Overview of the *CYP2B6* gene, allele definition and sequence variation with a focus of the exon 5 region

(A) Depicts the nine *CYP2B6* exons (gray boxes) with the most prominent SNPs and their respective amino acid changes. Nucleotide numbering corresponds to the *CYP2B6* cDNA. As highlighted by red bars, 785A>G (K262R) occurs in multiple haplotypes (*CYP2B6**4, *6, *7, *13, *16, *19 and *20). Other alleles including *CYP2B6**5, *9 and *18 lack 785A>G. Notably, the majority of SNPs are present in two or more alleles. The 777C>A SNP defining the *CYP2B6**3 haplotype is shown in blue. Other SNPs on alleles defined through the absence or presence of 785A>G are shown as black bars. The *CYP2B6*-specific XL-PCR product, utilized to confirm HRM obtained on gDNA, is denoted by a gray horizontal line. (B) Magnifies exon 5 and the 70 bp amplicon utilized for HRM analysis. Sequence variations differing from *CYP2B6**1 (wt) are highlighted by colored capital letters; respective amino acid changes are as shown. (C) Shows the *CYP2B7* exon 5 region that is simultaneously amplified with *CYP2B6* in the HRM assay when the assay is performed on gDNA. Note that *CYP2B6* carries a wild-type 'G' at the position that corresponds to *CYP2B6* 785A>G (bold). A minor *CYP2B7* variant carries a SNP at position 779C>T (position number equivalent to position in *CYP2B6*). HRM: High-resolution melt; wt: Wild-type; XL: Long range.

Figure 2. High-resolution melt normalization and differential plots depicting all *CYP2B6/CYP2B7* genotype combinations observed in the investigated sample panel

To aid visualization, **A & C**: HRM normalization plots and **B & D**: differential plots, each highlight four diplotypes in color (the remaining are shown in black) that were generated in a single run. Color codes are provided in **A & C** and match those utilized in **B & D**. The *CYP2B6/CYP2B7* diplotype IDs are listed in Table 2.

Figure 3. High-resolution melt normalization and differential plots depicting the four *CYP2B6* genotypes when the assay was performed on the preamplified *CYP2B6*-specific long-range PCR amplicons

(A) High-resolution melt (HRM) normalization and (B) differential plots. The color codes for the diplotypes shown are provided in the insert. The sample heterozygous for 769G>A and 785A>G (Table 2 diplotype ID 8) had the same melt profile as those homozygous for 785G. Samples with diplotypes differing only in *CYP2B7* are not distinguished because the preamplified long-range PCR template only represents *CYP2B6*; hence the samples representing diplotypes 1, 4, 2 and 5, respectively, exhibit identical HRM curves.

Table 1

Summary of primers for PCR and sequence analysis.

5'-3'	Used for	PCR product length
F: CACAGTGTGGAGAAGCAC R: GCAGGTAGGTGTCGATGAG	HRM [†]	70 bp
F: TCGGTCTGCCCATTCTATAAA R: CCTGCACTCACTTGCAATGT	XL-PCR	10,328 bp
F: ACAGAAGGATGAGGGAGGAA R: CTCTCCCTCTGTCTTTCATTCTGT	<i>CYP2B6</i> exon 5 sequencing	641 bp
F: ACAGAAGGATGAGGGAGGAA R: CACCACCACTCTCTCTCCA	<i>CYP2B7</i> exon 5 sequencing	608 bp

[†]This primer pair simultaneously amplifies a portion of *CYP2B6* and *CYP2B7* exon 5 as shown in detail in Figure 1.

F: Forward; HRM: High-resolution melt; R: Reverse; XL: Long range.

Table 2

CYP2B6 and CYP2B7 exon 5 diplotype combinations observed in 235 tissue samples.

Diplotype ID	Haplotype index	CYP2B7	779	CYP2B6			Caucasian (n)	African-American (n)	Unknown/other (n)	Total (n)
				769	777	785				
1	1	C		G	C	A	56	14	35	105
	1	C		G	C	A				
2	1	C		G	C	A	49	23	25	97
	2	C		G	C	G				
3	2	C		G	C	G	8	5	5	18
	2	C		G	C	G				
4	1	C		G	C	A	1	5	0	6
	3	T		G	C	A				
5 [†]	2	C		G	G	G	0	4	1	5
	3	T		G	C	A				
6	3	T		G	C	A	0	2	0	2
	3	T		G	C	A				
7	1	C		G	C	A	0	1	0	1
	4	C		G	A	A (*3)				
8 [‡]	2	C		G		G	0	0	1	1
	5	C		A		A (novel)				
Total n:							114	54	67	235

769G>A, 777C>A and 785A>G are SNPs within CYP2B6 and 779C>T is located in CYP2B7 (see Figure 1 for additional details). Variant nucleotides are shown in bold. Haplotype index see Table 3. Diplotype ID refers to the combination of alleles, or haplotypes, in a subject.

The diplotype IDs of the Coriell control samples are as following: NA17151 (ID 2), NA17244 (ID 2), NA17131 (ID 3), NA17124 (ID 3), NA17131 (ID 5) and NA17131 (ID 5).

[†]Based on data shown in Table 3, the more likely haplotype combination is shown.

[‡]This haplotype was confirmed by sequencing and performing high-resolution melt on a preamplified CYP2B6-specific long-range PCR product.

Table 3

Frequencies of inferred and observed *CYP2B6/CYP2B7* haplotypes.

Haplotype index	<i>CYP2B7</i> 779	<i>CYP2B6</i>			Frequency estimated (%)	Frequency observed (%)	Allele designation
		769	777	785			
1	C	G	C	A	67.16	66.81	wt
2	C	G	C	G	29.23	29.57	*4, *6, *7, *13, *16, *19, *20
3	T	G	C	A	2.96	3.19	wt 2B6 with 2B7 C>T
4	C	G	A	A	0.21	0.21	*3
5	C	A	C	A	0.10	0.21	Novel haplotype
6	C	A	C	G	0.11	0.00	NA
7	T	G	C	G	0.23	0.00	NA

The three *CYP2B6* and one *CYP2B7* SNPs (for details see Figure 1 & Table 2) are part of multiple defined or novel haplotypes as indicated. Two hypothetical haplotypes have not been observed.
NA: Not applicable; wt: Wild-type.