Development and Characterization of a Rapid and Comprehensive Genotyping Assay to Detect the Most Common Variants in Cytochrome P450 2D6

Lisa A. Scarlett,[1] Soraya Madani,[1] Danny D. Shen,[1] and Rodney J. Y. Ho[1,2]

Received September 21, 1999; accepted November 10, 1999

KEY WORDS: CYP2D6, genotyping assay, rapid and comprehensive, PCR

INTRODUCTION

The enzyme cytochrome P450 2D6 (CYP2D6) metabolizes a vast array of clinically important therapeutic agents including β-blockers, neuroleptics, tricyclic-antidepressants, and analgesics (1,2). About 3–10% of the Caucasian population exhibits low CYP2D6 activity and are "poor metabolizers" (PMs) (3). Individuals exhibiting CYP2D6 activity in a normal range are labeled "intermediate" or "extensive metabolizers" (IMs or EMs, respectively). PMs may experience unpleasant side-effects associated with elevated drug levels because of their slow rates of metabolic clearance of CYP2D6 substrates. Alternatively, PMs may experience therapeutic failure of CYP2D6 substrates that are pharmacologically inactive until they are converted to the active metabolites. Therefore, it is of interest to identify PMs prior to the administration of potentially toxic therapeutic agents. Identification can be accomplished by phenotyping with CYP2D6-specific substrates such as dextromethorphan or debrisoquine (4–6). Phenotyping can be relatively time consuming and exposes subjects to an unneeded drug. Recent advances in molecular techniques have led to improved, efficient, and non-invasive methods of PM identification.

The genetic basis of the CYP2D6 PM phenotype has been well characterized with almost 50 different mutations in the gene sequence described to date. However, greater than 98% of the PM phenotypes can be accounted for by five point mutations and a deletion of the entire CYP2D6 gene (7). Recently, Stüven *et al.* (8) employed a multiplex allele-specific PCR-based genotyping assay to simultaneously detect both mutant and wild-type alleles at five of these loci. Unfortunately, this assay did not detect the deletion mutation that represents approximately 17% of the PM phenotype (7). Therefore, the goal of this study is to develop a rapid and comprehensive genotyping assay that simultaneously detects both the deletion mutation and the remaining 5 major point mutations. In addition to detecting the mutated alleles, this assay also detects the wild-type alleles that will provide an internal control. Thus, the assay provides both a positive and negative outcome for each of the allelic variants of interest in a rapid and comprehensive manner.

MATERIALS AND METHODS

Tissue Procurement and Isolation of Nucleic Acids

Human liver samples were obtained from the University of Washington Solid Organ Transplant Program. All procedures were carried out with approval of the University of Washington Institutional Human Subjects Review Board. Donor characteristics ($n = 22$) are shown in Table 1. Livers were harvested from brain-dead donors and were rejected for transplantation for a variety of reasons, the most common being a high degree of fatty infiltration. Harvested livers were perfused with Belzer-University of Wisconsin cold storage solution and kept on ice until they were dissected into small pieces. The tissue pieces were then stored at −80°C for long-term preservation.

Genomic DNA was isolated from the samples using a Qiagen Tissue DNA Kit (Qiagen, Santa Clarita, CA) and the purified DNA was quantitated spectrophotometrically.

Genotyping

The detection of point mutations was based on the method of Stüven *et al.* (8). The CYP2D6 gene (4.7 kb) was amplified using primers PA-U and PA-L (Table 1) with the use of GeneAmp XL PCR kit containing rTth DNA polymerase (Perkin Elmer, Foster City, CA). The reaction mixture contained primers, 15 μl 3.3X XL buffer, 2.4μl $Mg(OAc)_2$ solution, 1μl rTth DNA polymerase and 200 μM of each dNTP, sterile nuclease-free H_2O, and 250 ng genomic DNA. The final volume was 50 μl. All samples were amplified in duplicate and the assay was repeated at least twice. Thermocycling was conducted in a Perkin Elmer DNA Thermocycler model 2400 using standard thin-walled reaction tubes (Island Scientific, Bainbridge Island, WA). The cycling conditions were as follows: hot start and initial denaturation at 94°C for 1 minute, 16 cycles of 94°C for 15 seconds and 68°C for 10 minutes, followed by 12 cycles of 94°C for 15 seconds and 68°C for 10 minutes with a 15 second incremental increase in annealing time per cycle. This was followed by a final extension of 10 minutes at 72°C. The 4.7 kb products were visualized in a 0.8% agarose gel with ethidium bromide staining.

Using each 4.7 kb CYP2D6 gene product as the DNA template, two separate allele-specific amplifications were performed. The two different reactions contained primers to amplify either wild-type or mutant alleles (Table 1). The reaction mixtures (50 μl total volume) contained 2.0 μl of template DNA (diluted 1:10 in Tris-EDTA), 200 μM each dNTP, 3.0 mM $MgCl_2$, 4.0 units Taq polymerase (Promega, Madison, WI, USA), primers, 5.0 μl Thermo DNA Poly 10X reaction buffer, and nuclease-free H_2O. All samples were amplified in duplicate and the assay was repeated at least twice. Thermocycling was performed in the Perkin Elmer 2400 thermocycler as follows: initial denaturation at 94°C for 2 minutes followed by 20 cycles of 94°C for 1 minute, 60°C for 40 seconds, and 72°C for 5 minutes. This was followed by a final extension at 72° for 7 minutes. Products were analyzed in a 3.0% agarose gel with ethidium bromide staining.

[1] Department of Pharmaceutics, University of Washington, Seattle, Washington 98195-7610.

[2] To whom correspondence should be addressed. (e-mail: rodneyho@u.washington.edu)

0724-8741/00/0200-0242$18.00/0

Table 1. Primer Sequences and Concentrations used in PCR for Determination of Genotype

Name	Used to detect	Sequence (5′ to 3′)	Final conc. (nM)
PA-U	2D6 gene product	GGT AAG GGC CTG GAG CAG GAA	300
PA-L	2D6 gene product	GCC TCA ACG TAC CCC TGT CTC	300
M	common upstream	GTG GGG CTA ATG CCT T	4000
A	2637 A	GGG TCC CAG GTC ATC CT	144
A*	2637 C (* 3 mutation)	GGG TCC CAG GTC ATC CG	72
B	1934 G	CGA AAG GGG CGT CC	80
B*	1934 A (*4 mutation)	CGA AAG GGG CGT CT	800
E	3023 A	GCT GCA CAT CCG GAT	352
E*	3023 C (*7 mutation)	GCT GCA CAT CCG GAG	72
T	1975 T	CTC CTC GGT CAC CCA	480
T*	1975 G (*6 mutation)	CTC CTC GGT CAC CCC	36
G	1846 G	TCT GCC CAT CAC CCA CC	80
G*	1846 T (*8 mutation)	TCT GCC CAT CAC CCA CA	360
FWD-D	*5 (gene deletion) mutation	ACC GGG CAC CTG TAC TGG TCA	300
REV-D	*5 (gene deletion) mutation	GCA TGA GCT AAG GCA CCC AGA C	300

The CYP2D6*5 (gene deletion) allele was detected by previously described method (9) with the use of the PE GeneAmp XL PCR Kit (Perkin Elmer, Foster City, CA, USA). Briefly, primers FWD-D and REV-D (final concentration for both was 0.3 μM, Table 1) were mixed with 15 μl 3.3X XL buffer, 200 μM each dNTP, 2.4 μl $Mg(OAc)_2$ solution, 1 μl rTth DNA polymerase, 250 ng genomic DNA, and sterile nuclease-free H_2O to a final volume of 50 μl. Thermocycling was carried out in parallel with amplification of the 4.7 kb CYP2D6 gene product. All samples were prepared in duplicate and the assay repeated at least twice. Products were visualized in a 0.8% agarose gel stained with ethidium bromide.

In Vitro CYP2D6 Activity Determination

Microsomes from respective livers were prepared using standard techniques (10) and *in vitro* CYP2D6 activity was determined by metoprolol α-hydroxylation as previously described (11). Metoprolol (20 μM) and its α-hydroxy metabolites were separated by HPLC and detected by a fluorescence monitor.

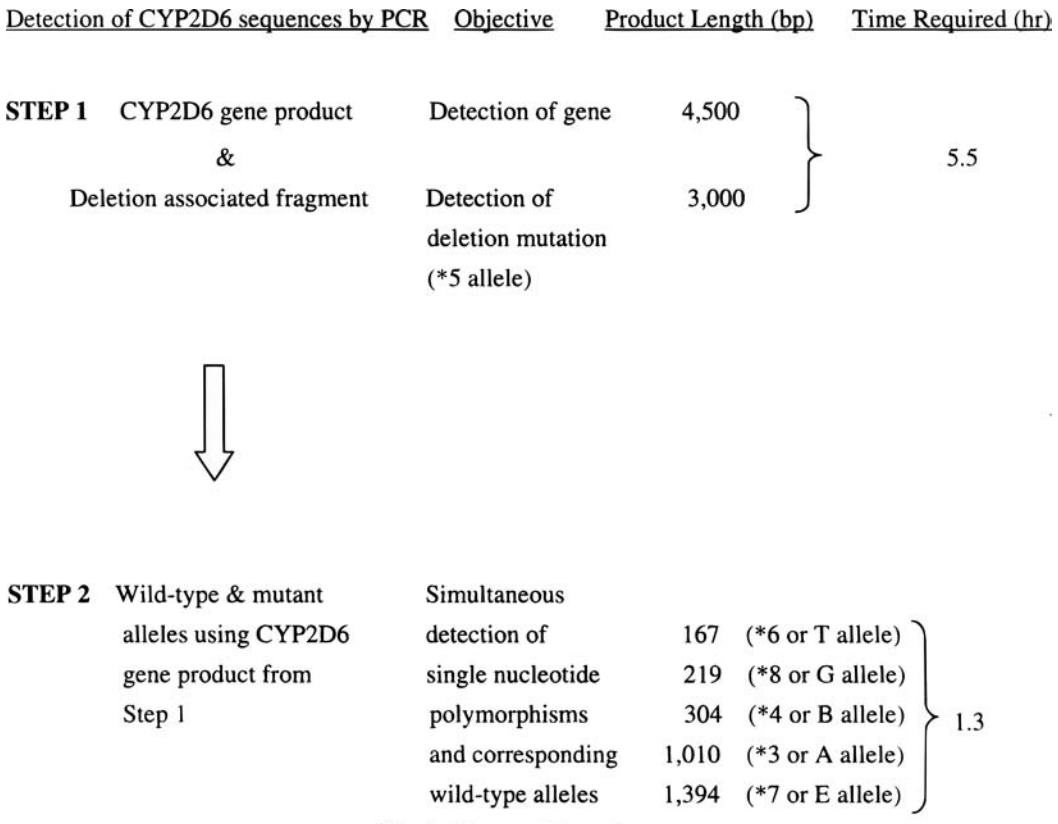

Fig. 1. Representation of assay.

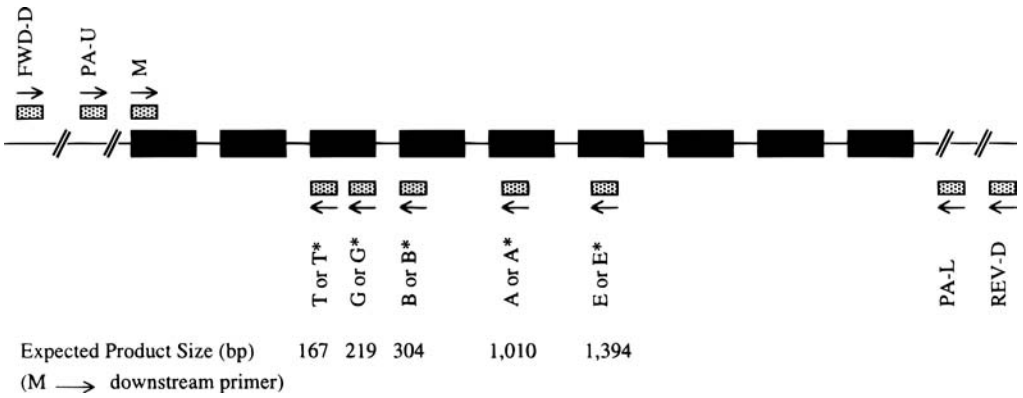

Fig. 2. CYP2D6 gene and location of primers used for PCR. The expected product size from the second-step PCR is indicated below the gene. The products result from amplification with M (common upstream primer) and the associated downstream primer.

RESULTS AND DISCUSSION

The goal of this study was to develop and characterize a simultaneously rapid and comprehensive genotyping assay for CYP2D6. Schematic representations of this method are shown in Figures 1 and 2. The first step involved the simultaneous amplification of the entire CYP2D6 gene and the deletion-associated fragment. The choice of polymerase used for this first step was critical. This is because amplified sequences longer than 3 kb (4.5 and 3.5 kb, respectively) were needed and any errors incurred in amplification by a non-proofreading enzyme would not be acceptable in the second step allele-specific PCR. Therefore, rTth DNA polymerase, designed specifically for long PCR applications, was used. This enzyme is a recombinant blend of polymerases from *Thermococcus litoralis* (Vent DNA polymerase) and *Thermus thermophilus*. The enzyme mixture was formulated with an optimized ratio of 5′−3′ polymerase activity to 3′–5′ exonuclease activity (12,13). The net result is an enzyme mix that provides a high degree of fidelity over fairly long amplification templates. In the present assay, the first amplification step was completed in 5.5 hours in a reliable and consistent manner. While utilizing long PCR technology took slightly longer than conventional genotyping methods, such an assay design was necessary to achieve the required level of fidelity in the sequence of our amplicons, used subsequently as templates.

The simultaneous amplification of both the entire CYP2D6 gene and deletion-associated fragment was critical as it imparted an internal control to the method. This allowed internal validation of the assay by detecting at least one positive band in either of the two amplification reactions (wild-type or deletion-associated). Anticipated results and agarose gel interpretation were as follows: one band in the gene product lane only—subject does not have the deletion mutation; one band each in the gene product lane and deletion-associated fragment lane—subject has one copy of the gene deletion mutation (*5); or one band only in the deletion-associated fragment lane—subject has both copies of the gene deleted.

Using the CYP2D6 gene product from the wild-type reaction above as the DNA template for the second step, we then screened for the remaining 5 major mutations following a previously described method (8). This approach, too, had an internal control. Both wild-type and variant sequences were detected using two separate reaction mixtures allowing a full genotypic characterization in a single PCR amplification step. Therefore, if a product appeared in the mutant panel for a given allele but was simultaneously absent in the corresponding wild-type

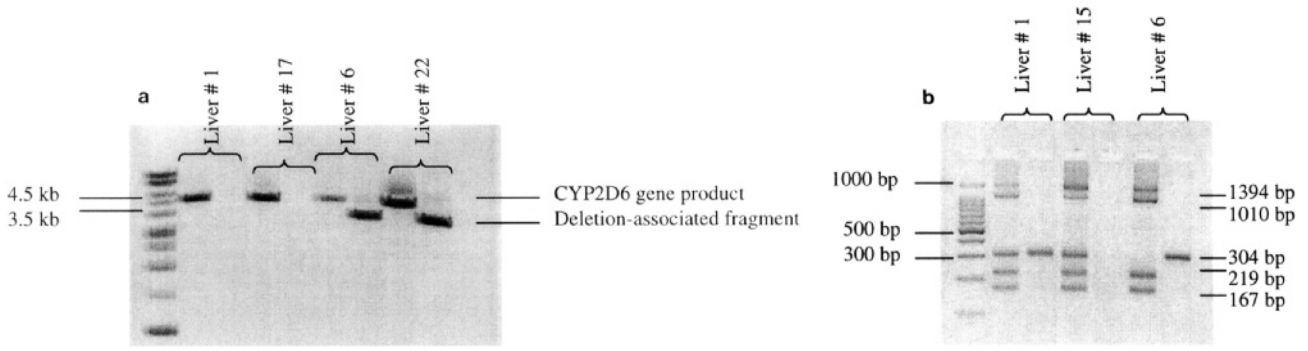

Fig. 3. (a) Representative detection and resolution of the first-step PCR products in a 0.8% agarose gel. The CYP2D6 gene product is 4.5 kb and the deletion-associated fragment is 3.5 kb. The first lane contains a 1 kb molecular weight ladder. (b) Representative detection and resolution of the second-step PCR products in a 3.0% agarose gel. The expected product sizes are indicated on the right side of the image. The first lane contains a 100 bp molecular weight ladder. Lanes 2, 4, and 6 are wild-type sequence reaction products and lanes 3, 5, and 7 are mutant sequence reaction products.

panel, the subject was homozygous for the mutant allele and classified as a PM. If, however, there were products in both the mutant and wild-type panels for a given allele, the subject was heterozygous at that locus. No product in the mutant panel but a corresponding product in the wild-type panel, again for a given allele, would indicate that the subject was homozygous wild-type at that locus. Because of the simplicity of this assay design and the relatively short PCR fragments to be generated, the second-step reaction can be completed in 1.3 hours. Therefore, the net result was that both first and second steps were completed in less than 8 hours or a typical workday.

Once both steps had been refined with respect to primer concentrations, cycling conditions, and enzyme choice, the method produced consistent results. Typical agarose gels containing the CYP2D6 gene product, deletion-associated fragment, and second step products are shown in Figures 3(a) and (b). From these images it is clear that the detection and resolution of the various alleles was accomplished relatively easily. With this optimized assay, we genotyped 22 human liver samples. A summary of genotypes obtained and donor characteristics are given in Table 2. The allelic frequencies for the more common *4 and *5 variants were 22% and 9%, respectively, similar to previously published findings (7). Given our limited sample number, we did not expect to find any of the less common mutant alleles in our bank.

To gain further insight into the relationship between genotype and phenotype, we compared the genotypes obtained to CYP2D6 activity determined in the liver microsome preparations. This activity was determined with metoprolol α-hydroxylation as the CYP2D6-specific probe. This drug exhibits moderately high turnover in human liver and has been used to study CYP2D6-mediated metabolism (11, 14). The results for *in vitro* activity determination of the 22 livers are given in Table 2. There was marked inter-individual variability with a broad range of activities (3.38–118.18 pmol/min/mg). The mean activity for * 1/*1 genotypes was 37.27 pmol/min/mg (±32.27) and the mean activity for livers with a *1/*4 genotype was 18.95 pmol/min. Livers with at least one variant allele had approximately 30% lower enzyme content, relative to homozygous wild-type livers, and those homozygous for mutations had no detectable enzyme. However, while there is a trend towards reduction in CYP2D6 activity with the *1/*4, *4/*5, and *1/*5 genotypes, it is not possible to draw firm conclusions regarding a definable relationship between genotype and phenotype due to the limited sample size.

The advent of high-throughput microarray-based chip technology has enabled the screening for multiple single-nucleotide polymorphisms in a single sample. This method is relatively comprehensive, but to our knowledge, current chip configurations do not allow for the unambiguous detection of the CYP2D6 gene deletion, which represents a significant percentage of PMs. Furthermore, this type of technology remains cost prohibitive for the use in routine applications. The chip itself, on a per subject basis, is expensive and the analysis of the hybridization results requires equipment that is also quite costly (15). Other technologies in development such as MALDI-TOF mass spectrophotometry for DNA sequencing, also require

Table 2. Liver Donor Characteristics, Genotypes and Phenotypes of Selected Samples from the University of Washington Liver Bank

Liver #	Donor age (yr)	Donor race	Donor gender	(deletion) *5	*3	*4	*6	*7	*8	Genotype	*In Vitro* enzymatic activity (pmol/min/mg)		CYP2D6 content (pmol/mg protein)	
9	7	Unknown	M							*1/*1	118.18		27	
16	59	Caucasian	M							*1/*1	93.74		41	
10	45	Caucasian	F							*1/*1	59.90		26	
17	48	Caucasian	M							*1/*1	53.38		28	
8	45	Caucasian	F							*1/*1	43.82		18	
5	49	Caucasian	M							*1/*1	35.65		21	
7	50	Caucasian	F							*1/*1	28.12		24	
14	15	Unknown	F							*1/*1	24.34	mean 37.27	15	mean 17.80
12	11	Black	M							*1/*1	22.23	stdev 32.27	13	stdev 9.54
21	60	Caucasian	F							*1/*1	20.80		9	
18	68	Caucasian	F							*1/*1	20.71		12	
20	70	Caucasian	F							*1/*1	17.42		10	
15	63	Caucasian	M							*1/*1	11.74		6	
19	37	Caucasian	M							*1/*1	5.70		14	
13	9	Caucasian	F							*1/*1	3.38		3	
3	51	Caucasian	M			x				*1/*4	25.63		9	
2	38	Caucasian	M			x				*1/*4	22.67		24	
1	10	Caucasian	F			x				*1/*4	19.54	mean 18.95	9	mean 12.20
11	39	Caucasian	M			x				*1/*4	15.38	stdev 5.63	9	stdev 6.61
4	36	Caucasian	F			x				*1/*4	11.51		10	
22	63	Caucasian	F	x						*1/*5	4.54		0	
6	62	Caucasian	F	x		x				*4/*5	4.32		0	

* n = 22 Allelic Frequency 9% 22%

equipment that is cost prohibitive and are not amenable to the rapid determination of genotype.

However, some of the methods described in the current study could be automated and adaptated to new microarray designs, which allows high-throughput screening. Detection limits can also be improved with the use of mass spectophotometric detection of PCR products.

In summary, we have developed and characterized an internally validated, comprehensive genotyping assay for the 6 most common mutations of CYP2D6 that can be completed within 8 hours. Most importantly, this assay method incorporates an internal control in its design and is easily adaptable to incorporate new variant alleles as they become known. In both steps, gene amplification and allele specific PCR, the assay is designed to detect both the wild-type sequence and corresponding mutant sequence, if present. Thus, it is a robust, reproducible, time and cost conscious method to confirm the heterozygous or homozygous character of an individual.

ACKNOWLEDGMENTS

Supported in part by NIH grants GM 32165, AI 31854, HL 56548. LS is supported by GM 07750 and the William E. Bradley Fellowship. The authors also wish to thank Brian Rasmussen for his critical review of this manuscript.

REFERENCES

1. S. Chen, W. H. Chou, R. A. Blouin, Z. Mao, L. L. Humphries, Q. C. Meek, J. R. Neill, W. L. Martin, L. R. Hays, and P. J. Wedlund. The cytochrome P450 2D6 (CYP2D6) enzyme polymorphism: screening costs and influence on clinical outcomes in psychiatry, *Clin. Pharmacol. Ther.* **60**:522–534 (1996).
2. P. Dalen, M. L. Dahl, M. L. Bernal Ruiz, J. Nordin, and L. Bertilsson. 10-hydroxylation of nortriptyline in white persons with 0, 1, 2, 3, and 13 functional CYP2D6 genes, *Clin. Pharmacol. Ther.* **63**:444–452 (1998).
3. C. Sachse, J. Brockmoller, S. Bauer, and I. Roots. Cytochrome P450 2D6 variants in a Caucasian population: allele frequencies and phenotypic consequences, *Am. J. Hum. Genet.* **60**:284–295 (1997).
4. D. Köhler, S. Härtter, K. Fuchs, W. Sieghart, and C. Hiemke. CYP2D6 genotype and phenotyping by determination of dextromethorphan and metabolites in serum of healthy controls and of patients under psychotropic medication, *Pharmacogentics.* **7**:453–461 (1997).
5. F. J. Gonzalez and J. R. Idle. Pharmacogenetic phenotyping and genotyping: present status and future potential, *Clin. Pharmacokinet.* **26**:59–70 (1994).
6. A. K. Daly, M. Armstrong, S. C. Monkman, M. E. Idle, and J. R. Idle. Genetic and metabolic criteria for the assignment of debrisoquine 4-hydroxylation (Cytochrome P4502D6) phenotypes, *Pharmacogenetics.* **1**:33–41 (1991).
7. D. Marez, M. Legrand, N. Sabbagh, J. M. Lo Guidice, C. Spire, J. J. Lafitte, U. Meyer, and F. Broly. Polymorphism of the cytochrome P450 CYP2D6 gene in a European population: characterization of 48 mutations and 53 alleles, their frequencies and evolution, *Pharmacogenetics.* **7**:193–202 (1997).
8. T. Stüven, E. U. Griese, H. K. Kroemer, M. Eichelbaum, and U. M. Zanger. Rapid detection of CYP2D6 null alleles by long distance- and multiplex-polymerase chain reaction, *Pharmacogenetics.* **6**:417–421 (1996).
9. V. M. Steen, O. A. Andreassen, A. K. Daly, T. Tefre, A. L. Børresen, J. R. Idle, and A. K. Gulbrandsen. Detection of the poor metabolizer-associated CYP2D6(D) gene deletion allele by long-PCR technology, *Pharmacogenetics.* **5**: 215–223 (1995).
10. M. F. Paine, M. Khalighi, J. M. Fisher, D. D. Shen, K. L. Kunze, C. L. Marsh, J. D. Perkins, and K. E. Thummel. Characterization of inter- and intra-intestinal differences in human CYP3A-dependant metabolism, *J. Pharmacol. Exp. Ther.* **283**:1552–1562 (1997).
11. S. Madani, M. F. Paine, L. Lewis, K. E. Thummel, and D. D. Shen. Comparison of CYP2D6 content and metoprolol oxidation between microsomes isolated from human livers and small intestines, *Pharm. Res.* **16**:1199–1205 (1999).
12. W. M. Barnes. PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates, *Proc. Natl. Acad. Sci. USA* **91**: 2216–2220 (1994).
13. S. Cheng, C. Fockler, W. M. Barnes, and R. Higuchi. Effective amplification of long targets from cloned inserts and human genomic DNA, *Proc. Natl. Acad. Sci. USA* **91**:5695–5699 (1994).
14. S. V. Otton, H. K. Crewe, M. S. Lennard, G. T. Tucker, and H. F. Woods. Use of quinidine inhibition to define the role of the sparteine/debrisoquine cytochrome P450 in metoprolol oxidation by human liver microsomes, *J. Pharmacol. Exp. Ther.* **247**:242–247 (1998).
15. M. Johnston. Gene chips: array of hope for understanding gene regulation, *Curr. Biol.* **8**:R171–174 (1998).