CYP2D6 Genotyping as an Alternative to Phenotyping for Determination of Metabolic Status in a Clinical Trial Setting

Submitted: July 5, 2000; Accepted: September 22, 2000; Published: October 29, 2000.

Suzin McElroy, Jodi Richmond, Maruja Lira, David Friedman, B. Michael Silber, and Patrice M. Milos

Pharmacogenomics and Clinical Biochemical Measurements, Discovery Research, Pfizer Global Research and Development, Eastern Point Road, Groton, CT 06340

Christoph Sachse, Jürgen Brockmöller, and Ivar Roots

Institute of Clinical Pharmacology, University Medical Centre Charite', Humboldt University of Berlin, Schumannstr. 20/21, 10117 Berlin, Germany

ABSTRACT The emerging application of pharmacogenomics in the clinical trial setting requires careful comparison with more traditional phenotyping methodologies, particularly in the drug metabolism area where phenotyping is used extensively. The research objectives of this study were 1) to assess the utility of *cytochrome P450 2D6 (CYP2D6*) genotyping as an alternative to traditional phenotyping as a predictor of poor metabolizer status; 2) to identify issues for consideration when implementing *CYP2D6* genotyping in clinical trials; and 3) to outline the advantages and disadvantages of *CYP2D6* genotyping compared with phenotyping. DNA samples obtained from 558 previously phenotyped individuals were blindly genotyped at the*CYP2D6* locus, and the genotype-phenotype correlation was then determined. The *CYP2D6*genotyping methodology successfully predicted all but 1 of the 46 poor metabolizer subjects, and it was determined that this 1 individual had a novel (presumably inactive) mutation within the coding region. In addition, we identified 2 subjects with *CYP2D6* genotypes indicative of poor metabolizers who had extensive metabolizer phenotypes as determined by dextromethorphan/dextrorphan ratios. This finding suggests that traditional phenotyping methods do not always offer 100% specificity. Our results suggest that *CYP2D6* genotyping is a valid alternative to traditional phenotyping in a clinical trial setting, and in some cases may be better. We also discuss some of the issues and considerations related to the use of genotyping in clinical trials and medical practice.

Corresponding Author: Patrice M. Milos, Pharmacogenomics, Genomic and Proteomic Sciences, Discovery Research, Pfizer Global Research and Development, Eastern Point Road, Groton, CT 06340; telephone: 860-441-4342; facsimile: 860-441-0436; e-mail: Patrice_M_Milos@Groton.Pfizer.com

Key Words: Cytochrome P450 2D6, Pharmacogenomics, Drug Metabolism, Genotyping.

Introduction

Cytochrome P450s comprise the major enzymes involved with Phase I metabolism of xenobiotics. One of these enzymes, cytochrome P450 2D6 (CYP2D6), processes about 20% of all commonly prescribed drugs, and many compounds currently in clinical development are known to be CYP2D6 substrates. This enzyme has a wide range of activity within human populations, with interindividual rates of metabolism differing more than 10 000-fold (1-4). This variation poses a challenge to drug development as it results in difficulty in predicting dosing, safety, and efficacy. Most individuals are able to metabolize CYP2D6 substrates extensively(extensive metabolizers [EMs]), whereas 7% to 10% of Caucasian individuals produce no functional CYP2D6 enzyme and are forced to use an alternative metabolic pathway (poor metabolizers [PMs]). A small percentage of individuals exhibit either a rate of metabolism between that of EMs and PMs (intermediate metabolizers [IMs]) or an ultrarapid rate of metabolism (ultrarapid metabolizers [UMs]); ultrarapid metabolization may be a result of multiple tandem copies of the

CYP2D6 gene in one individual. Because of such large potential differences in pharmacokinetics, clinicians are at an advantage if they know the metabolic status of the subjects enrolled in clinical studies for drugs known to be metabolized by CYP2D6, and often attempts are made to determine a subject's metabolic status before administration of a drug in clinical trials. Better decisions regarding drug dosing, safety, and efficacy can be made with knowledge of the subject's CYP2D6 status.

CYP2D6 metabolic status has traditionally been determined by administering a probe drug that is known to be a CYP2D6-specific substrate, such as dextromethorphan or debrisoquine. Urine samples are then collected at a certain time point, and the ratio of unchanged drug to metabolite is determined by high-performance liquid chromatography or gas chromatography (5, 6). Although this phenotyping methodology is reliable, some problems are associated with it. Intraindividual variability may be significant, and the length of time it takes to determine metabolic status delays administration of the test drug. Careful drug screens must be used because there may be significant drug-drug interactions with CYP2D6 inhibitors or inducers that can lead to inaccurate metabolic measurements (7, 8). Finally, the rate of metabolism of the probe drug may not be a reflection of that of the test drug.

Several studies have shown that much of the interindividual variation found in CYP2D6 metabolic activity is a result of genetic polymorphisms within the *CYP2D6* gene (1, 4, 9-18). Alleles that result in increased, decreased, or no enzyme activity have been identified, characterized (9, 10, 12, 19, 20), and used to support studies investigating the use of genotyping to predict the metabolic status of an individual (3, 21-24). Genotyping could have major advantages over phenotyping in that intraindividual variability is not an issue and results can be obtained more quickly. Current genotyping methodologies are simple polymerase chain reaction (PCR)-based assays that require only a small amount of whole blood from a patient, and the techniques used are easily adaptable in any molecular biology laboratory.

With the emerging interest in the field of pharmacogenomics, we feel that *CYP2D6*genotype-phenotype correlation could serve as an early validation of the increasingly important role that human genetics will play in the clinical development and marketing of therapeutics (15). We assessed the utility of *CYP2D6* genotyping as an alternative or replacement to the traditional.6 phenotyping methods by genotyping 558 patients who had previously been metabolically characterized through phenotyping.

Materials and Methods

Genomic DNA was isolated from whole blood from each of the 558 subjects, and the *CYP2D6* genotype was determined using a modified version of the methodology described by Sachse et al (3) (see Appendix 1). The methodology uses several PCR assays to screen for single nucleotide polymorphisms within the *CYP2D6* gene as well as gene duplication and deletion (25) and pseudogene hybrid formation (19). Results from the individual assays were used to determine the ultimate *CYP2D6* genotype. Table 1 shows the mutations that were screened for and their presence in each of the established *CYP2D6* alleles, of which at least 18 are known. To determine the *CYP2D6* genotype, data from each of the assays were examined collectively and compared with Table 1. Some of the alleles share several point mutations, and therefore each individual mutation detected was accounted for. It was important to distinguish between heterozygotes and homozygotes, as homozygotes have two identical mutations, one on each chromosome strand, whereas heterozygotes have the mutation on only one strand. For example, the alleles *2, *10,

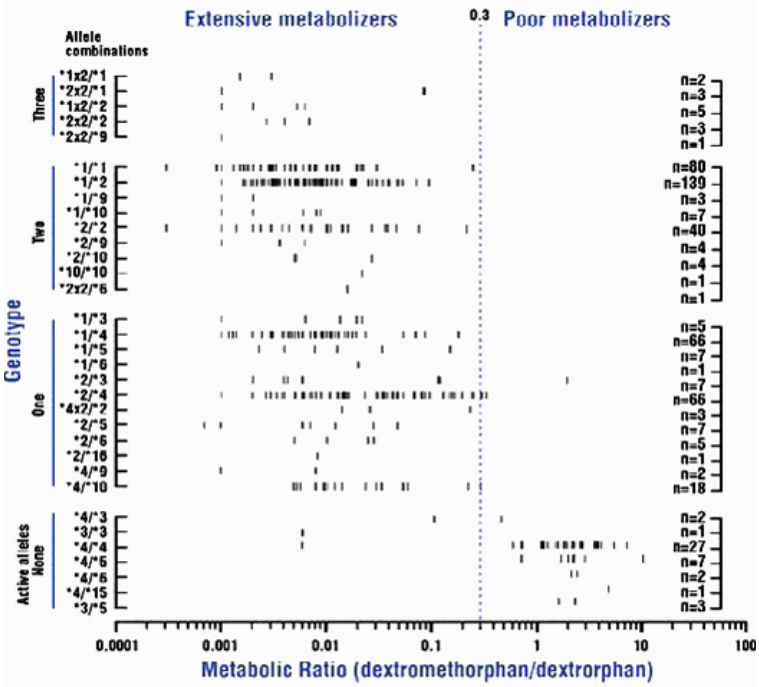
Table I
Defined genotypes of 18 known alleles of the *CYP2D6* gene.

	Del 1795	G 1934 A	G 1846 T	G 1846 A	Del A 2637	*5 (2D6 del)	C 188 T	G 212 A	Ins T 226	A 3023 C	G 971 C	*9	C 2938 T	G 4268 C	G 1749 C	*16 hybrid	2D6 dup
*1																	
*2													x	x	x		
*3					x												
*4		x					x							x	x		
*5						x											
*6	x																
*7										x							
*8			x										x	x	x		
*9												x	x				
*10							x							x	x		
*11											x		x	x	x		
*12								x					x	x	x		
*14				x			x						x	x			
*15									x								
*16																x	
*1x2																	x
*2x2													x	x	x		x
*4x2		x					x							x	x		x

*9 is defined as a deletion of nucleotides A2701-A2703 or G2702-A2704

and *4 share the point mutations G4268C and G1749C, and *10 and *4 share C188T. Thus, a patient sample that had detected mutations of G1934A, C188T, C4268C, C2938T, and C1749C would have a genotype of *2/*4, while a patient sample that had detected mutations of C188T, C4268C, C2938T, and C1749C would have a genotype of *2/*10. The ultimate *CYP2D6* genotype, which is the combination of the two alleles (one on each chromosome) detected in the sample, can be represented by a classification system that considers the number of functional alleles in the genotype when making phenotypic predictions (3). One could possess 0, 1, 2, or 3+ functional alleles depending on the genotype, as in Figure 1. In this classification system, functional alleles include *1, *2, *9, *10, and *17. Nonfunctional alleles include *3, *4, *5, *6, *7, *8, *11, *12, *13, *14, *15, *16, and *4XN. Individuals with a *1xN or *2xN could be considered to possess additional functional alleles dependent on the number of tandem copies of the gene.

Figure 1.



Plot of *CYP2D6* genotype versus phenotype for 222 patients from Pfizer clinical studies.
CYP2D6 genotype was determined as outlined in Methods section.
CYP2D6 phenotype was measured using the logarithm of the ratio of detromethorphan/dextrorphan (DM/DX) in urine.
Genotypes are categorized as having zero, one, two, or three active alleles.
An arbitrary cutoff of 0.3 for DM/DX separates extensive metabolizers (EMs), in which DM/DX<0.3, from poor metabolizers (PMs), in which DM/DX>0.3.

The PM, EM, UM, and IM groups are not well defined for either phenotype or genotype. Typically, the arbitrarily placed cutoff between EM and PM is 0.3 for the plasma ratio of dextromethorphan substrate to the dextrorphan metabolite and 12.6 for the debrisoquine/4-hydroxydebrisoquine plasma ratio (26). There is, however, no arbitrarily defined cutoff for UM/EM, EM/IM, and IM/PM phenotype borders. Thus, we have loosely termed phenotypes in the range of <0.001 to 0.08 as EM, 0.08 to 0.3 as IM, and >0.3 as PM for dextromethorphan/dextrorphan; and <0.05 to 6 as EM, 6 to 12.6 as IM, and >12.6 as PM for debrisoquine/4-hydroxydebrisoquine. We chose not to include UM as a category at this time. In most cases, an EM or PM distinction would probably suffice, but we were also interested in investigating the utility of the IM category..8

Results and Discussion

Each of the 558 subjects whom we genotyped had been previously phenotyped using either dextromethorphan or debrisoquine, thereby allowing us to investigate genotype/phenotype correlation and ability to predict metabolic status from genotype. All but 2 of the subjects who had a

CYP2D6 genotype containing no functional alleles had a PM phenotype. These 2 exceptions were confirmed by DNA sequencing to have *4/*4 and *3/*4 genotypes, which are indicative of PMs, yet the DM/DX ratios were 0.062 and 0.1078, respectively. This suggests an inaccurate measure of phenotype because no functional CYP2D6 enzyme would be present in these individuals. Each of the patients with one or more functional alleles, with 1 exception, had an EM or IM phenotype. This 1 exception had a calculated genotype of *2/*3 (1 functional allele) and a PM phenotype of metabolic ratio equal to 1.962. Each of the *CYP2D6* exons and exon-intron boundaries from this patient were sequenced, and a novel polymorphism was detected. Because this polymorphism results from a nucleotide insertion in exon 9, we suspect that it contributed to the PM status of the patient (manuscript in preparation).

Table 2 shows the polymorphisms that were interrogated and whether they were detected in any of the 558 patients analyzed. Several of the PM alleles (*7, *8, *11, *12, and *14) were not detected

Table II
Polymorphism of the *Cyp2D6* gene screened for and detected in 558 patients

Polymorphisms screened for	Enzyme Activity in vivo	in 558 samples?	Association with 2D6 alleles
Del T1795	none	Y	*6
G1934A	none	Y	*4,*4xN
G1846T	none	N	*8
G1846A	none	N	*14
Del A2637	none	Y	*3
2D6 gene deletion	none	Y	*5
C188T	reduced	Y	*4,*10,*14,*4xN
G212A	none	N	*12
Ins T226	none	Y	*15
A3023C	none	N	*7
G971C	none	N	*11
Del A2701-A2703 or G2702-A2704	slightly reduced	Y	*9
C2938T	slightly reduced	Y	*2,*8,*9,*11, *12,*14,*2xN
G4268C	slightly reduced	Y	*2,*4,*8,*10, *11,*12,*14,
G1749C	slightly reduced	Y	*2,*4,*8,*10,*11, *12,*2xN,*4xN
2D6/2D7P hybrid	none	Y	*16
2D6 gene duplication	enhanced or none	Y	*1xN,*2xN,*4xN

Table III A and B
(A) *CYP2D6* genotype distribution among poor metabolizer subjects in 222 Pfizer clinical patients and 336 Humboldt patients (alone and combined). There were 46 PM subjects total.
(B) *CYP2D6* allelic contribution to the poor metabolizer genotypes

A. Genotype Distribution Among Poor Metabolizer Subjects

Genotype	N, Pfizer (freq)	N, Humboldt (freq)	N, combined (freq)
*4/*4	11 (0.58)	19 (0.70)	30 (0.652)
*4/*5	4 (0.21)	4 (0.15)	8 (0.174)
*3/*5	3 (0.16)	0 (0)	3 (0.065)
*4/*6	0 (0)	2 (0.07)	2 (0.043)
*4/*15	0 (0)	1 (0.04)	1 (0.022)
*3/*4	0 (0)	1 (0.04)	1 (0.022)
*3/novel	1 (0.05)	0 (0)	1 (0.022)

B. Allelic Contribution to Poor Metabolizer Genotypes

Allele	Freq in 19 PM subjects (Pfizer)	Freq in 27 PM subjects (Humboldt)	Freq in 46 PM subjects (combined)
*3	0.1053	0.0185	0.0543
*4	0.6842	0.8519	0.7826
*5	0.1842	0.0741	0.1196
*6	0	0.037	0.0217
*15	0	0.0185	0.0109
novel	0.0263	0	0.0109

in any of the 558 samples (1116 chromosomes). Table 3A outlines the genotype distribution among PM subjects, and Table 3B details the allelic contribution to PM genotypes. The PM alleles *3, *4, and *5 accounted for more than 95% of the contribution to PM genotypes, but the extended screen that included the less common PM alleles (those with a frequency of less than 0.001) was able to detect an additional 4 PM subjects who would have been missed if we had performed genotyping for only the most common alleles. The extended screen did not, however, detect the novel polymorphism in exon 9 of one subject, which presumably contributed to that subject's PM status. Finally, one of the PM alleles, *16, was not found in any of the PMs, yet it was detected in 1 EM subject with a genotype of *1/*16. It is important to note that a rare PM allele, when present, may not necessarily contribute to a PM phenotype as long as it is paired with a functional allele, as PM status is a recessive condition.

The ability of molecular genetics to correlate with drug exposure is an early validation of the emerging field of pharmacogenomics. In this study and others (3, 23),

CYP2D6 genotyping has proven to be a valid alternative to traditional phenotyping for determination of poor metabolizer status. Given the correlation between *CYP2D6* genotype and phenotype, we believe that *CYP2D6* genotyping should be used in the clinical trial setting. In our study we identified two individual phenotypic measurements that were predictive of subjects who would have exhibited an extensive metabolizer status yet were genotyped as poor metabolizers, which was then confirmed by DNA sequencing of genomic DNA from the subjects. Therefore, in the context of these clinical development studies, traditional phenotyping did notoffer 100% specificity. This discrepancy could have resulted from sample handling or data reporting errors or assay failure. We were unable to go back to the individual subjects to reanalyze the phenotype.

Genotyping methodologies can be easier to use than biochemical measurements in a clinical setting. Genotyping requires only a single sample, whereas biochemical measurements often require various body fluids and/or tissue biopsies taken at multiple time points. Often a very small amount of material, such as blood from a single finger prick or a buccal swab, is all that is needed to complete genotyping assays. Genotyping results can often be obtained more quickly than phenotyping results, and future technologies may offer rapid "bedside" assessment. For assessment of CYP2D6 status, samples for genotyping can be obtained at the screening visit, and results can be obtained before the wash-out period is over; phenotyping usually cannot be performed until after the wash-out period. Because an individual's DNA sequence does not change over time, genotyping for a particular gene needs only to be performed once, whereas biochemical measurements may need to be taken numerous times. Finally, genotyping is likely to be less expensive,

especially as new high-throughput technologies become available.

CYP2D6 genotyping methodology is well documented and should be easy for any molecular biology laboratory to adapt. Genotyping should yield the same results across different laboratories, thus making it ideally suited to a diagnostic application (30); in contrast, phenotyping may yield more variable data between laboratories and at different time points for the same patient. Intraindividual variation does not exist in genotype data; on any given day a patient's genotype will be exactly the same. This constancy does not apply to CYP2D6 phenotypes because other factors, such as certain foods, tobacco usage, and drug-drug interactions, can act as CYP2D6 inducers or inhibitors and thus give an incorrect measure of CYP2D6 metabolic rate upon administration of the probe drug. This fact could be particularly important in clinical trials of neurotrauma drugs in which the patient may be unconscious. The nature of these compounds and their ability to penetrate the blood-brain barrier make them susceptible to CYP2D6 metabolism, and if the patient is unconscious, a physician may have no knowledge of drugs that the patient may have been taking that are CYP2D6 inducers or inhibitors (Table 4). Even though in such cases genotyping would give an accurate prediction of metabolic status (while phenotyping might not), a physician still needs to be aware of other factors that can introduce variability in CYP2D6 metabolism.

CYP2D6 genotyping is also a flexible methodology in that one can decide which alleles to interrogate depending on the needs of each particular study. The advantage of screening for all known alleles is to increase our ability to safely test new therapeutic entities. However, screening for the most common PM alleles (*3, *4, *5) will typically identify about 95% of the PMs in a sample set. There are, however, several other PM alleles that result in a complete loss of enzyme activity, some of which are extremely rare. For example, *13, which was not included in this study because we had no positive control group, may have an allele frequency as low as 0.0001 (19). Not including a screen for rare alleles may result in a missed PM. In addition, screening for alleles that result in a decreased enzyme function (particularly *10), as opposed to no enzyme function, may be beneficial. For example, Asian populations are known to have a higher allelic frequency of the *10 allele (27-29), which could help explain the overall lower capacity of Asian patients to metabolize CYP2D6 substrates. Although the genotypic prediction of IM status was correlated with an IM phenotype only 10% of the time in our study, the use of the IM category can be quite useful in Phase I trials in which the safety and efficacy windows are unknown. In addition, some alleles share several polymorphisms, thereby offering a quality check on the determined genotype. For example, the *4 allele has not only G1934A, but also shares C188T,

Table IV
Drugs that interact with CYP2D6 enzyme activity *in vivo*

Therapeutic Category	Drug Type	Drug Name
Antiarrhythmics	Type 1A	quinidine
		disopyramide
		procainamide
	Type 1B	mexiletine
	Type 1C	encainide
		flecainide
		propafenone
Antidepressants	Non-tricyclic	bupropion
		maprotiline
		trazodone
	Tricyclic	amitriptyline
		amoxapine
		clomipramine
		desipramine
		doxepin
		imipramine
		nortriptyline
		protriptyline
		trimipramine
	SSRI	fluoxetine
		nefazodone
		paroxetine
		sertraline
		venlafaxine
Analgesics	Narcotics	codeine
		hydrocodone
		dihydrocodeine
		propoxyphene
		tramadol
Antipsychotics	Phenothiazines	prochlorperazine
(neuroleptics)		promethazine
		chlorpromazine
		thioridazine
	Other	risperidone
	Other	haloperidol
Anticonvulsants		carbamazepine
		phenytoin
		gabapentin
Antihistamines	Tricyclic	loratadine
Antihypertensive Agents	Beta-Blockers	metoprolol
		pindolol
		propanolol
		timolol
Stimulants	Sympathomimetic	methamphetamine
Other medications		cimetidine
		metoclopramide
		dextromethorphan
		capsaicin

G4268C, and G1749C with *10. Thus, if G1934A is the only mutation detected, one might hesitate to claim that the genotype contains a *4 allele. One strategy for increasing genotyping efficiency might be to develop a protocol that includes an initial screen for the more common PM alleles (*3, *4, *5, *6, and *16) and, in cases where none of these alleles are detected, a screen for the less common PM alleles and IM alleles.

CYP2D6 genotyping may be helpful in drug-drug interaction studies of novel therapeutics. In such studies where there is interpatient variability in safety or efficacy, one might conclude that there is some kind of relevant interaction between the two administered drugs and that this interaction is responsible for the observed variability. However, examination of the *CYP2D6* genotype (and/or those of other drug-metabolizing enzymes) may reveal that the variability was attributable to poor metabolizer status in those patients in whom variability was observed. We have used *CYP2D6* genotyping in several drug-drug interaction studies in which a test drug was co-administered with dextromethorphan or paroxetine, two drugs known to be metabolized by CYP2D6, and concluded that the interpatient variability observed was indeed attributable to poor metabolizer status.

Prospective *CYP2D6* genotyping for enrollment purposes may be extremely useful in Phase I first-in-human (FIH) studies where safety and efficacy profiles are essentially unknown. For safety purposes, a clinician may initially choose to enroll only subjects with 2 active alleles. Genotype can be compared with data on pharmacokinetic variability, adverse events, and drug response to aid in the decision-making process for subsequent studies for the compound, eventually including PM individuals. This information is important for all drugs in development, no matter which metabolizing enzymes are involved, as it could help make clinical trials safer with respect to drug metabolism. In clinical trials in which significant variability in drug response or pharmacokinetic data are observed, retrospective genotyping is probably easier to use and more informative than retrospective phenotyping. A further benefit of genotyping is that the comparison of genotype and pharmacokinetic data is likely to indicate an individual's rate of metabolism for a particular drug, whereas the rates of metabolism of probe drug and test drug may be significantly different in a phenotype screen.

CYP2D6 genotyping is likely to assist clinicians in finding drug doses that are safe and efficacious for both EMs and PMs, or perhaps in tailoring drug dosage to an individual's needs, with EMs and PMs receiving differential dosing.

Although genotyping has many advantages over phenotyping in a clinical trial setting, it also has some limitations. First, the described PCR-RFLP methodology has some technical shortcomings. For example, some of the assays are prone to false negatives because they rely on the presence or absence of a PCR product. In addition, the RFLP banding patterns and band intensities may be confusing (see Appendix 2). Using this methodology, one could potentially miss some PMs because a rare polymorphism or a novel functional polymorphism was not screened for. This illustrates one major limitation of

CYP2D6 genotyping by the methodology described. By screening only for known *CYP2D6* polymorphisms, PMs with novel or rare polymorphisms that result in no enzyme activity could be missed. However, in screening 1116 chromosomes we identified only one novel allele that contributed to a PM phenotype; therefore, the PCR-RFLP methodology should be reliable more than 99.5% of the time. Second, even though genotype is an excellent predictor of *CYP2D6* poor metabolizer status, it is probably not an absolute predictor of catalytic function (22). Patients of a single common genotype have DX/DM ratios over a 1000- to 10 000-fold range, yet there is a significant gene-dose effect for both debrisoquine and dextromethorphan for all PM-IM-EM-UM differences (Figure 1). The precise reason for this range is unknown but is probably due to diet, differential expression of the *CYP2D6* gene, inherent backup metabolism systems, significant interlaboratory and intraindividual variability in phenotype data, and possible combinations of other minor undetected genetic polymorphisms in the *CYP2D6* gene (28). *CYP2D6* genotyping could, therefore, be challenging for therapies with narrow therapeutic indices, as

intermediate metabolizers cannot be readily identified. Third, specific drug-drug interactions can convert extensive metabolizers to poor metabolizers. While this is a minor issue in the clinical trial setting where concombinant medicines can be carefully screened, drug-drug interactions in a traditional medical care setting can have extremely important consequences because patients can be far more difficult to monitor. *CYP2D6*genotyping would be of little use in such cases where it is a drug-drug interaction, not metabolic status, that is problematic. A final limitation of genotyping is the ethical issue surrounding anonymous and non-anonymous genotyping. Patients may feel that genetic studies are an invasion of their privacy, and some may worry that employers or insurance companies could get access to genotype data. However, genotyping of the drug-metabolism genes may present fewer ethical issues because, generally, these genes have not been associated with increased risk for disease. Still, for legal and ethical reasons, genotyping in clinical trials requires new consent forms and institutional review board approval.

While genotyping may become an integral part of clinical development, its future in medical practice is less certain. The field of pharmacogenomics will provide an explosion of genetic data that could potentially have an enormous impact on drug development from discovery research through postmarketing. Discovery research in pharmacogenomics can enhance our ability to establish the therapeutic relevance of novel candidate genes in humans through genetic association studies that reveal novel associations with particular human phenotypes. In early clinical development, the assessment of polymorphic drug-metabolizing enzyme genotypes in all Phase I studies will aid in a comprehensive understanding of pharmacokinetic variability. Additionally, the investigation of therapeutic target variation early in development will better define patient subpopulations and potentially enhance our understanding of efficacy and safety. In Phase III studies, large patient populations will allow the possible identification of those subpopulations where efficacy and safety issues are imperative for drug approval. Similarly, issues arising postmarketing could be addressed with postmarketing collection of DNA samples and subsequent pharmacogenomic analyses.

How the pharmaceutical industry will use this wealth of information is uncertain. Private companies and regulatory agencies alike will have to determine the value of pharmacogenomics in the marketplace as opposed to in the clinical trial setting. From a drug metabolism standpoint, genetic data may never be as informative as drug level monitoring, but if the purpose is simply to prevent poor metabolizers from pursuing a particular therapy due to safety issues, a simple diagnostic genotyping assay may be required for prescriptions to be written. Although a push for diagnostic genotyping would likely complicate drug development issues, we should strive to maintain the same high standards in the marketplace that we do in the clinical trial setting. The choice of whether to use genotyping in diagnostic assays will probably initially be made on a case-by-case basis and will be driven by the sensitivity and specificity of the genetic diagnostic, the cost/benefit of such a diagnostic, and the perceived need.

Conclusions

In conclusion, *CYP2D6* genotyping is a valid alternative to traditional phenotyping, and in many cases genotyping is uniquely well suited to the clinical trial setting. Pharmacogenomics is a rapidly evolving area with many technological advances that will increase throughput and decrease costs for genotyping assays. In many respects the

CYP2D6 gene represents a challenge for genotyping because the numerous polymorphisms are not only single nucleotide in nature, but are also gene deletion, duplication, and pseudogene derivatives. Yet this genetic information promises to optimize drug therapy by potentially decreasing the number of adverse events through individualized dosing dependent on a patient's *CYP2D6* genotype. Future technologies that might be used for *CYP2D6* genotyping in the patient setting include TaqMan allelic discrimination (eg, PerkinElmer-Applied Biosystems, Wellesley, MA) DNA chip-based assays for specific alleles (eg, Affymetrix, Santa Clara, CA), or MALDI-TOF mass spectrometric methods (eg, Sequenom, San Diego, CA). All of these technologies, however, have the limitation that they can only detect known single nucleotide

polymorphisms. Given the large amount of genetic diversity at the *CYP2D6* locus, it is likely that there are more novel mutations that result in a loss of enzyme activity. Ideally, the emerging genotyping technologies will be able to detect these rare mutations so that no poor metabolizers would be missed. The ideal *CYP2D6*genotyping technology would be a high-throughput sequencing platform that allows an individual's entire *CYP2D6* gene to be rapidly sequenced. The establishment of pharmacogenomic service laboratories associated with CROs will greatly facilitate the incorporation of genotyping in clinical trials. One can imagine a day when the use of genotyping to determine CYP2D6 metabolic status, as well as the status of other important polymorphic drug-metabolizing enzymes, may be an integral part of patient care and therapeutic management.

Acknowledgments

C.S. and J.B. were supported by BMBF grants 01EC9408 and 01ZZ9511. I.R. and J.B. thank BMBF grant 01GG9845/5. The authors wish to thank Dr Albert Seymour, Dr Phil Chappell, and Beva Nall-Langdon for critical reading of the manuscript; Darcy Nelson for technical expertise; and Linda Nelms and Christopher Hinkel for initial *CYP2D6* genotyping experiments.

References

1. Kroemer HK, Eichelbaum M. Molecular basis and clinical consequences of genetic cytochrome P450 2D6 polymorphism. *Life Sciences.* 1995;56:2285-2298.

2. Nebert DW. Polymorphisms in drug-metabolizing enzymes: what is their clinical relevance and why do they exist? *Am J Hum Genet.* 1997;60:265-271.

3. Sachse C, Brockmöller J, Bauer S, Roots I. Cytochrome P450 2D6 variants in a caucasian population: allele frequencies and phenotypic consequences. *Am J Hum Genet.* 1997;60:284-295.

4. West WL, Knight EM, Pradhan S, Hinds TS. Interpatient variability: genetic predisposition and other genetic factors. *J Clin Pharmacol.*1997;37:635-648.

5. Lennard MS, Silas JH, Smith AJ, Tucker GT. Determination of debrisoquine and its 4-hydroxy metabolite in biological fluids by gas chromatography with flame ionization and nitrogen-selective detection. *J Chromatogr.*1977;133:161-166..19

6. Chen ZR, Somogyi AA, Bochner F. Simultaneous determination of dextromethorphan three metabolites in plasma and urine using high-performance liquid chromatography with application to their disposition in man. *Ther Drug Monit.*1990;12:97-104.

7. Köhler D, Härtter S, Fuchs K, Sieghart W, Hiemke C. *CYP2D6*genotype and phenotyping by determination of dextromethorphan and metabolites in serum of healthy controls and of patients under psychotropic medication.*Pharmacogenetics.*1997;7:453-461.

8. Özdemir V, Naranjo CA, Herrmann N, Reed K, Sellers EM, Kalow W. Paroxetine potentiates the central nervous system side effects of perphenazine: contribution of cytochrome P450 2D6 inhibition *in vivo. Clin Pharmacol Ther.* 1997;62:334-347.

9. Gonzalez FJ, Skoda RC, Kimura S, et al.Characterization of the common genetic defect in humans deficient in debrisoquine metabolism. *Nature.* 1988;331:442-446.

10. Gough AC, Miles JS, Spurr NK, et al. Identification of the primary gene defect at the cytochrome P450 CYP2D locus. *Nature.* 1990;347:772-775.

11. Saxena R, Shaw GL, Relling MV, et al. Identification of a new variant

CYP2D6 allele with a single base deletion in exon 3 and its association with the poor metabolizer phenotype. *Hum Mol Genet.* 1994;3:923-926..20

12. Daly AK, Brockmöller J, Broly F, et al. Nomenclature for human *CYP2D6* alleles. *Pharmacogenetics.* 1996;6:193-201.

13. Sachse C, Brockmöller J, Bauer S, Reum T, Roots I. A rare insertion in exon 1 of *CYP2D6* causes a frameshift and is associated with the poor metabolizer phenotype:*CYP2D6*15*. *Pharmacogenetics.* 1996;6:269-272.

14. Yokoi T, Kosaka Y, Chida M, et al. A new *CYP2D6* allele with a nine base insertion in exon 9 in a Japanese population associated with poor metabolizer phenotype.*Pharmacogenetics.* 1996;6:395-401.

15. Linder MW, Prough RA, Valdes R Jr. Pharmacogenetics: a laboratory tool for optimizing therapeutic efficiency *Clin Chem.* 1997;43:254-266.

16. Løvlie R, Daly AK, Idle JR, Steen VM. Characterization of the 16+9 kb and 30+9 kb *CYP2D6* XbaI haplotypes. *Pharmacogenetics.* 1997;7:149-152.

17. Marez D, Legrand M, Sabbagh N, et al. Polymorphism of the cytochrome P450 CYP2D6 gene in a European population: characterization of 48 mutations and 53 alleles, their frequencies and evolution. *Pharmacogenetics.* 1997;7:193-202..21

18. Oscarson M, Hidestrand M, Johansson I, Ingelman-Sundberg M. A combination of mutations in the *CYP2D6*17 (CYP2D6Z)* allele causes alterations in enzyme function. *Mol Pharmacol.* 1997;52:1034-1040.

19. Daly AK, Fairbrother KS, Andreassen OA, London SJ, Idle JR, Steen VM. Characterization and PCR-based detection of two different hybrid *CYP2D7P/CYP2D6* alleles associated with the poor metabolizer phenotype. *Pharmacogenetics.* 1996;6:319-328.

20. Chen S, Chou W-H, Blouin RA, et al. The cytochrome P450 2D6 (CYP2D6) enzyme polymorphism: screening costs and influence on clinical outcomes in psychiatry. *Clin Pharm Ther.* 1996;60:522-534.

21. Madsen H, Hansen TS, Brøsen K. Imipramine metabolism in relation to the sparteine oxidation polymorphism a family study. *Pharmacogenetics.* 1996;6:513-519.

22. Griese E-U, Zanger UM, Brudermanns U, et al. Assessment of the predictive power of genotypes for the catalytic function of CYP2D6 in a German population. *Pharmacogenetics.* 1998;8:15-26.

23. Sachse C, Brockmöller J, Hildebrand M, Müller K, Roots I. Correctness of prediction of the CYP2D6 phenotype confirmed by genotyping 47 intermediate and poor metabolizers of debrisoquine. *Pharmacogenetics.* 1998;8:181-185.

24. Brockmöller J, Kirchheiner J, Meisel C, Roots I. Pharmacogenetic diagnostics of cytochrome P450 polymorphisms in clinical drug development and in drug treatment. *Pharmacogenomics.* In press.

25. Johansson I, Lundqvist E, Dahl M-L, Ingelman-Sundberg M. PCR-based genotyping for duplicated and deleted *CYP2D6* genes. *Pharmacogenetics.* 1996;6:351-355.

26. Schmid B, Bircher J, Preisig R, Küpfer A. Polymorphic dextromethorphan metabolism: co-segregation of oxidative O-methylation with debrisoquine hydroxylation. *Clin Pharmacol Ther.* 1985;38:618-624.

27. Johansson I, Oscarson M, Yue Q-Y, Bertilsson L, Sjöqvist F, Ingelman-Sundberg M. Genetic analysis of the Chinese *cytochrome P4502D* locus: characterization of variant*CYP2D6* genes present in subjects with diminished capacity for debrisoquine hydroxylation.*Mol Pharmacol.* 1994;46:452-459.

28. Wang S-L, Lai M-D, Lai M-L, Huang J-d. R296C and other *CYP2D6* mutations in Chinese.

Pharmacogenetics. 1995;5:385-388..23

29. Aynacioglu AS, Sachse C, Bozkurt A, et al. Low frequency of defective alleles of cytochrome P450 enzymes 2C19 and 2D6 in Turkish population. *Clin Pharmacol Ther.* 1999; 66:185-192.

30. Kashuba ADM, Nafziger AN, Kearns GL, et al. Quantification of intraindividual variability and the influence of menstrual cycle phase on CYP2D6 activity as measured by dextromethorphan phenotyping.

Pharmacogenetics. 1998;8:403-410.

31. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manua*l. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1974.

32. Steen VM, Andreassen OA, Daly AK, et al. Detection of the poor metabolizer-associated *CYP2D6(D)* gene deletion allele by long-PCR technology. *Pharmacogenetics.* 1995;5:215-223.

33. Douglas AM, Atchison BA, Somogyi AA, Drummer OH. Interpretation of a simple PCR analysis of *CYP2D6(A)* and *CYP2D6(B)* null alleles associated with the debrisoquine/sparteine genetic polymorphism. *Pharmacogenetics.* 1994;4:154-158..24

Appendix 1. *CYP2D6* Genotyping Methodology

Subjects and Methods

Subjects

All the studies in which whole blood was obtained were approved by the appropriate institutional human experimentation committees in accordance with all applicable regulations. Three hundred thirty-six unrelated Caucasian German individuals described previously (3) and 222 patients from various Pfizer clinical trials (primarily unrelated Caucasian males) were studied for CYP450 2D6 metabolic status with appropriate informed consent.

Phenotyping Methods

Each of the 558 subjects was phenotyped for their CYP450 2D6 metabolic status. Of the German individuals, 302 were phenotyped with dextromethorphan and 34 with debrisoquine, as described (3). Each of the Pfizer subjects was phenotyped with dextromethorphan (6).

Genotyping Methods.

Genomic DNA was isolated from whole blood from each of the 558 subjects using either the QIAmp Blood Kit (QIAGEN, Valencia, CA) (Pfizer samples) or the method of Sambrook et al (31) (German samples). The 336 German samples were first genotyped in Berlin as described (3) and then genotyped using a modified methodology described below at Pfizer in Groton, CT, for a cross-laboratory comparison of *CYP450 2D6* genotyping methodology. The 222 Pfizer samples were also genotyped using this modified methodology.

Minor modifications were made to the established methodology of Sachse et al (3). All PCR reactions were performed in PerkinElmer MicroAmp Optical plates (96 well) using either Perkin Elmer GeneAmp PCR System 9600 or 9700 thermocyclers. All PCR reagents were from Perkin-Elmer. Restriction enzymes were from New England BioLabs (Beverly, MA), except for MaeII, which was from Boehringer Mannheim. SeaKem GTG and MetaPhor agarose for gel electrophoresis were both from FMC BioProducts (now a subsidiary of Cambrex, East Rutherford, NJ). Table 5 outlines the *CYP2D6* genotyping methodology used at Pfizer.

Results from the individual assays were used to determine the ultimate *CYP2D6* genotype. Table 1 shows the mutations that were screened for and their presence in each of the established *CYP2D6* alleles. To determine the *CYP2D6* genotype, data from each of the assays were examined collectively and compared with Table 1. It was important to distinguish between heterozygotes and homozygotes, as homozygotes have two identical mutations, one on each chromosome strand, whereas heterozygotes have the mutation on only one strand. Some of the alleles share several point mutations, and therefore each individual mutation detected was accounted for. For example, the alleles *2, *10, and *4 share the point mutations G4268C and G1749C, and *10 and *4 share C188T. Thus, a patient sample that had detected mutations of G1934A, C188T, C4268C, C2938T, and C1749C would have a genotype of *2/*4, while a patient sample that had detected mutations of C188T, C4268C, C2938T, and C1749C would have a genotype of *2/*10.

Sequencing of Exons and Intron-Exon Boundaries

Initial amplification product was used as a template for nested PCR amplifications in which the products spanned exons and intron-exon junctions. Nested primers were tagged with M13 universal primers for ease of direct cycle sequencing. Primer sequences are shown in Table 6. Each 100 µL reaction contained 1X PCR Buffer II, 2.5 mmol/L $MgC^{l}2$, 0.2 mmol/L dNTPs, 0.2 µmol/L primers, 2.5 units of AmpliTaq Gold, and diluted initial amplification product. Cycling parameters on the 9600 were

95° for 10 minutes and 35 cycles of 95° for 30 seconds, 63° for 45 seconds, and 72° for 1 minute. PCR products were gel purified, and 5-10 ng were used in each DyePrimer Cycle Sequencing (PerkinElmer-Applied BioSystems) reaction. Samples were run on either ABI373 or ABI377, and sequences were analyzed using Factura and AutoAssembler software.

Appendix 2. Example of RFLP Diagnostic Fragments for *CYP2D6* Genotyping.

A major cause of genotype discrepancies is miscalled RFLPs at point mutations, namely C188T and G1934A. Figure 2 shows the results of the Reaction 1 BstNI digests of 44 samples. Example 1 is A1934A (homozygous mutant), and example 2 is G1934G (homozygous wild type). Example 3 has bands indicative of both G and A at 1934 and is heterozygous. However, example 4 also has bands indicative of both G and A at 1934, but the larger molecular weight band is much less intensely stained than the lower molecular weight bands; hence, there is some confusion about what the true genotype at that position is. We hypothesize that the reason for this phenomenon is unequal PCR amplification of the two strands that results in one strand being more abundant in the final product. Sequencing of exon 4 can quickly reveal the true genotype.

Table V
Outline of *CYP2D6* genotyping methodology.

Reaction	Detected Mutations	Primers (5' to 3')	Template DNA	PCR Enzyme	Cycling Conditions	PCR Product Length (bp)	Restriction Digest	Agarose gel type	Diagnostic Fragment Patterns	Reference Paper
Initial amplification	N/A	CCAGAAGGCTTTGCAGGCTTCA	gDNA	r *Taq*, XL	94° for 15 seconds	~5 kb	N/A	0.8% SeaKem GTG	N/A	30
		ACTGAGCCCTGGGAGGTAGGTA	(~100 ng)	(4 units)	62° for 5 minutes					
					(25 cycles)					
					autoextend for 15 seconds					
					(10 cycles)					
Reaction 3	2D6 gene deletion	ACCTGGGCACCTGTACTCCTCA	gDNA	r *Taq*, XL	94° for 15 seconds	~3.5 kb, if deletion is present	N/A	0.8% SeaKem GTG	N/A	31
		GCATGAGCTAAGGCACCCAGAC	(~100 ng)	(2 units)	66° for 4 minutes					
					(20 cycles)					
					autoextend for 15 seconds					
					(12 cycles)					
Reaction 12	2D6 gene multiplication	GCCACCATGGTGTCTTTGCTTTC	gDNA	r *Taq*, XL	94° for 15 seconds	~10 kb, if multiplication is present	N/A	0.6% SeKem GTG	N/A	30
		ACCGGATTCCAGCTGGGAAATG	(~100 ng)	(4 units)	65° for 8 minutes					
					(20 cycles)					
					autoextend for 15 seconds					
					(15 cycles)					
Reaction 11	2D6/2D8P hybrid	TGGGGCCCAAGGCAGGGACT	gDNA	AmpliTaq	94° for 30 seconds	~1.4 kb, if hybrid is present	N/A	1% SeaKem GTG	N/A	19
		TGCTCAGCCTCAACGTACCCC	(~100 ng)	(2.5 units)	66° for 10 seconds					
					72° for 90 seconds					
					(35 cycles)					
Reaction 1	Del T at 1795	CCTGGGCAAGAAGTCGCTGGACCAG	initial amplification	AmpliTaq Gold	95° for 10 minutes	353 bp	BstNI	2.5% MetaPhor	T/T-190,163	3
(nested)		GAGACTCCTCGGTCTCTCG	product	(2.5 units)	94° for 30 seconds				Del T/Del T-190,139,23	
			(diluted)		60° for 10 seconds				T/Del T-190,163,139,23	
					72° for 60 seconds					
	G1934A				(35 cycles)		BstNI		G/G-190,163	
									A/A-355	
									G/A-355,190,163	
	G1846T or A						MspI		G/G-278,75	
									T or A/T or A-353	
									G/T or A-353,278,75	
Reaction A	Del A2637	CGCCTTCGCCAACCAGTCCG	initial amplification	AmpliTaq Gold	95° for 10 minutes	835 bp	MseII	2.5% MetaPhor	A/A-352,329,154	32
		GGCTGGGCTGGGTCCCAGGTCATAC	product	(2.5 units)	94° for 30 seconds				Del A/Del A-352,303,154,26	
			(diluted)		56° for 10 seconds				A/Del A-352,329,303,154,26	
(nested)					72° for 60 seconds					
					(35 cycles)					
Reaction 4	C188T	TCAACACAGCAGGTTCA	initial amplification	AmpliTaq Gold	95° for 10 minutes	433 bp	HphI	2.5% MetaPhor	C/C-362,71	3
(nested)		CTGTGGTTTCACCCACC	product	(2.5 units)	94° for 30 seconds				T/T-262,100,71	
			(diluted)		52° for 10 seconds				C/T-362,262,100,71	
					72° for 60 seconds					
	G212A				(35 cycles)		MspI		G/G-242,141,50	3
									A/A-242,191	
									G/A-242,191,141,50	
	Ins T at 226						BspMI		T/T-157,156,120	8
							(NEBuffer 2)		Ins T/Ins T-278,156	
									T/Ins T-278,157,156,120	
Reaction 5	A3025C	CCCGTTCTGTCCCCAGTATG	initial amplification	AmpliTaq Gold	95° for 10 minutes	187 bp	FokI	2.5% MetaPhor	A/A-124,37,26	8
(nested)		GGGCTCACGCTGCACATCAGGA	product	(2.5 units)	94° for 30 seconds				C/C-124,63	
			(diluted)		60° for 10 seconds				A/C-124,63,37,26	
					72° for 60 seconds					
					(35 cycles)					
Reaction 6	G971C	TGCTCACTCCTGGTAGCC	initial amplification	AmpliTaq Gold	95° for 10 minutes	256 bp	PstI	2.5% MetaPhor	G/G-114,106,36	3
(nested)		AGGATCTGGGTGATGGGCA	product	(2.5 units)	94° for 30 seconds				C/C-142,114	
			(diluted)		56° for 10 seconds				G/C-142,114,106,36	
					72° for 60 seconds					
					(35 cycles)					
Reaction 7	C2938T	AGGCCTTCCTGGCAGAGATGAVG	initial amplification	AmpliTaq Gold	95° for 10 minutes	386 bp	BspMI + MboII	2.5% MetaPhor	C/C-230,124,32	3
(nested)		CCCCTGCACTGTTTCCCAGA	product	(2.5 units)	94° for 30 seconds		(NEBuffer 2)		T/T-354,32	
			(diluted)		60° for 10 seconds				C/T-354,230,124,32	
					72° for 60 seconds					
	Del A2701-A2703				(35 cycles)				no del/no del-230,124,32	3
	or								Del/Del-262,124	
	Del G2702-A2704								no del/Del-262,230,124,32	
Reaction 8	G4268C	CGTCTAGTGGGGAGACAAAC	initial amplification	AmpliTaq Gold	95° for 10 minutes	881 bp	BanII	2.5% MetaPhor	G/G-420,235,107,101,18	3
(nested)		CTCAGCCTCAACGTACCCCT	product	(2.5 units)	94° for 30 seconds				C/C-420,336,107,18	
			(diluted)		60° for 10 seconds				G/C-420,336,235,107,101,18	
					72° for 60 seconds					
					(35 cycles)					
Reaction 9	G1749C	TAATGCCTTCATGCCCACCCG	initial amplification	AmpliTaq Gold	95° for 10 minutes	467 bp	BsmAI	2.5% MetaPhor	G/G-311,156	8
(nested)		GAGACTCCTCGGTCTCTCG	product	(2.5 units)	94° for 30 seconds				C/C-209,156,102	
			(diluted)		60° for 10 seconds				G/C-311,209,156,102	
					72° for 60 seconds					
					(35 cycles)					
Reaction 12A	multiplicated	GCCACCATGGTGTCTTTGCTTTC	Reaction 12	AmpliTaq Gold	95° for 10 minutes	264 bp	Ban II	2.5% MetaPhor	*1XN-231,33	3
(nested - 12)	*1, *2, or *4 allele	CTCAGCCTCAACGTACCCCT	product	(2.5 units)	94° for 30 seconds				*2XN-264	
			(diluted)		52° for 10 seconds				*4XN-264	
					72° for 60 seconds					
					(35 cycles)					
Reaction 12B	multiplicated	TCAACACAGCAGGTTCA	Reaction 12	AmpliTaq Gold	95° for 10 minutes	433 bp	HphI	2.5% MetaPhor	*1XN-362,71	3
(nested - 12)	*1, *2, or *4 allele	CTGTGGTTTCACCCACC	product	(2.5 units)	94° for 30 seconds				*2XN-362,71	
			(diluted)		52° for 10 seconds				*4XN-262,100,71	
					72° for 60 seconds					
					(35 cycles)					

Table VI

Sequences of primers tagged with M13 universal primers for sequencing the exons of the *CYP2D6* gene.

Exon	Forward Primer	Reverse Primer
1	TGTAAAACGACGGCCAGTGAGCCCATTTGGTAGTGAGGCAGG	CAGGAAACAGCTATGACCCCTCTGCCGCCCTCCAGGACC
2	TGTAAAACGACGGCCAGTCTGGCTTGACAAGAGGCCCTGACC	CAGGAAACAGCTATGACCCGGAAATCTGTCTCTGTCCCCACC
3	TGTAAAACGACGGCCAGTCACGCGCACGTGCCCGTCCCAC	CAGGAAACAGCTATGACCAGTTCCCGCTTTGTGCCCTTCTGC
4	TGTAAAACGACGGCCAGTAGGCGACCCCTTACCCGCATCTCC	CAGGAAACAGCTATGACCCCTGCAGAGACTCCTCGGTCTCTC
5	TGTAAAACGACGGCCAGTAGGAGGGATTGAGACCCCGTTCTG	CAGGAAACAGCTATGACCCCACCGTGGCAGCCACTCTCACC
6	TGTAAAACGACGGCCAGTCGTTCTGTCCCGAGTATGCTCTCG	CAGGAAACAGCTATGACCCTCGGCCCCTGCACTGTTTCCCAG
7	TGTAAAACGACGGCCAGTGCTGACCCATTGTGGGGACGCATG	CAGGAAACAGCTATGACCTGCTGAGCTGGGGTGAGGAGGGCG
8	TGTAAAACGACGGCCAGTCAGTCCCCACTCTCACCCTGCATC	CAGGAAACAGCTATGACCGAAGGGGACAGGGAGCCGGGCTCC
9	TGTAAAACGACGGCCAGTAGCCAGGCTCACTGACGCCCCTCC	CAGGAAACAGCTATGACCTGATCCCAACGAGGGCGTGAGCAG