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The Development, Validation and Implementation of a Broad-Based ADME Genotyping Assay into Research and Clinical Trials

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Cette thèse intitulée:

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Résumé

Afin d'adresser la variabilité interindividuelle observée dans la réponse pharmacocinétique à de nombreux médicaments, nous avons créé un panel de génotypage personnalisée en utilisant des méthodes de conception et d'élaboration d'essais uniques. Celles-ci ont pour but premier de capturer les variations génétiques présentent dans les gènes clés impliqués dans les processus d'absorption, de distribution, de métabolisme et d'excrétion (ADME) de nombreux agents thérapeutiques. Bien que ces gènes et voies de signalement sont impliqués dans plusieurs mécanismes pharmacocinétiques qui sont bien connues, il y a eu jusqu'à présent peu d'efforts envers l'évaluation simultanée d'un grand nombre de ces gènes moyennant un seul outil expérimental. La recherche pharmacogénomique peut être réalisée en utilisant deux approches: 1) les marqueurs fonctionnels peuvent être utilisés pour présélectionner ou stratifier les populations de patients en se basant sur des états métaboliques connus; 2) les marqueurs Tag peuvent être utilisés pour découvrir de nouvelles corrélations génotype-phénotype. Présentement, il existe un besoin pour un outil de recherche qui englobe un grand nombre de gènes ADME et variantes et dont le contenu est applicable à ces deux modèles d'étude. Dans le cadre de cette thèse, nous avons développé un panel d'essais de génotypage de 3,000 marqueurs génétiques ADME qui peuvent satisfaire ce besoin. Dans le cadre de ce projet, les gènes et marqueurs associés avec la famille ADME ont été sélectionnés en collaboration avec plusieurs groupes du milieu universitaire et de l'industrie pharmaceutique. Pendant trois phases de développement de cet essai de génotypage, le taux de conversion pour 3,000 marqueurs a été amélioré de 83% à 97,4% grâce à l'incorporation de nouvelles stratégies ayant pour but de surmonter les zones d'interférence génomiques comprenant entre autres les régions homologues et les polymorphismes sous-jacent les régions d'intérêt. La précision du panel de génotypage a

été validée par l'évaluation de plus de 200 échantillons pour lesquelles les génotypes sont connus pour lesquels nous avons obtenu une concordance > 98%. De plus, une comparaison croisée entre nos données provenant de cet essai et des données obtenues par différentes plateformes technologiques déjà disponibles sur le marché a révélé une concordance globale de > 99,5%. L'efficacité de notre stratégie de conception ont été démontrées par l'utilisation réussie de cet essai dans le cadre de plusieurs projets de recherche où plus de 1,000 échantillons ont été testés. Nous avons entre autre évalué avec succès 150 échantillons hépatiques qui ont été largement caractérisés pour plusieurs phénotypes. Dans ces échantillons, nous avons pu valider 13 gènes ADME avec cis-eQTL précédemment rapportés et de découvrir et de 13 autres gènes ADME avec cis eQTLs qui n'avaient pas été observés en utilisant des méthodes standard. Enfin, à l'appui de ce travail, un outil logiciel a été développé, Opitimus Primer, pour aider pour aider au développement du test. Le logiciel a également été utilisé pour aider à l'enrichissement de cibles génomiques pour d'expériences séquençage. Le contenu ainsi que la conception, l'optimisation et la validation de notre panel le distingue largement de l'ensemble des essais commerciaux couramment disponibles sur le marché qui comprennent soit des marqueurs fonctionnels pour seulement un petit nombre de gènes, ou alors n'offre pas une couverture adéquate pour les gènes connus d'ADME. Nous pouvons ainsi conclure que l'essai que nous avons développé est et continuera certainement d'être un outil d'une grande utilité pour les futures études et essais cliniques dans le domaine de la pharmacocinétique, qui bénéficieraient de l'évaluation d'une longue liste complète de gènes d'ADME.

Mots-clés : La pharmacogénomique, la pharmacocinétique, ADME, le génotypage, le développement technologique

Abstract

In order to better assess the inter-individual variability observed in a patient's pharmacokinetic response to many medications, we have created a custom genotyping panel that uses unique assay designs to analyze variation present in key genes involved in the absorption, distribution, metabolism and excretion (ADME) of many therapeutic agents. These genes and pathways involved in most pharmacokinetic mechanisms are well known. However, as yet, there has been little effort to develop tools that can interrogate a large number of variations in most known drug metabolizing genes simultaneously within a single experimental tool. Pharmacogenomic research has historically been conducted using two approaches: targeted studies that screen a small number of specific functional markers to identify known metabolic status phenotypes, and genome-wide studies that identify novel genetic correlations with drug response phenotypes. Thus, a gap currently exists for a targeted ADME research tool that can evaluate a large number of key ADME genes and variants in a format that can be applicable to both types of study designs. As part of this thesis, we have developed a 3000 SNP broad based ADME genotyping panel that can address this need.

Genes and markers for the genotyping panel were selected in collaboration with many groups from both academia and the pharmaceutical industry in an effort to capture all pertinent genes and metabolic pathways that have been implicated in drug metabolism. The final assay design was composed of over 3000 markers in 181 genes. Over three phases of iterative development, the assay conversion rate for the 3000 markers was improved from 83.0% to 97.4% through the incorporation of novel design strategies to overcome areas of genomic interference such as regions of homology and underlying polymorphisms. Accuracy of the assay was validated by screening more than 200 samples of known genotype with a concordance of

99%. Additionally, data from the assay has also been compared to data from different technological platforms and has an overall concordance of 99.5%. The effectiveness of the design strategy was demonstrated in the successful utilization of the assay in the screening of over 1000 samples which identified several novel pharmacogenetic associations between ADME variations and adverse drug reactions in children. Another goal of this thesis was to demonstrate what added benefit/utility the 3000 SNP ADME panel would have when compared to currently available genotyping assays. Using 150 extensively investigated liver samples, the broad based assay was not only able to detect and validate 13 previously reported cis eQTLs in ADME genes but further identified an additional 13 novel ADME cis eQTLs that had never been observed before, doubling the number previously identified using standard methods on the same samples. Finally, in support of this work, a number of bioinformatic tools had to be developed to help expedite this research. These tools have been further refined and are currently being used to assist with enrichment of genomic targets for next generation sequencing experiments.

In conclusion, this work has led to a better understanding of ADME genetics and the nuances of assaying ADME genes. The content and designs of the developed assay sets it apart from currently available commercial assays that contain only functional markers in a small number of genes or do not have adequate coverage across ADME genes. The assay has the ability to play a significant role in pharmacogenomic studies to identify known and novel pharmacogenomic biomarkers. These will lead to improved biomarkers that will help better stratify pharmaceutical clinical trial populations or assist physicians to select better, more personalized, efficacious and safer therapies for their patients.

Keywords : Pharmacogenomics, pharmacokinetics, ADME, genotyping, technology development

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Abbreviations

6-MP	6-mercaptopurine
ABC	adenosine triphosphate-binding cassette
ACHE	acetylcholinesterase
ACS	acute coronary syndrome
ADH	alcohol dehydrogenase
ADME	absorption, distribution, metabolism and excretion
ADP	adenosine diphosphate
ADR	adverse drug reaction
AF-1	activation function 1 domain
AhR	aryl hydrocarbon receptor
ALDH	aldehyde dehydrogenase
Arnt	aryl hydrocarbon nuclear translocator
ASO	allele specific oligonucleotide
ATP	adenosine triphosphate
AZA	azathioprine
BCHE	butyrylcholinesterase
BCRP	breast cancer resistance protein
BBB	blood brain barrier
BMI	body mass index
CAR	constitutive androstane receptor
CES	carboxylesterase
CEU	CEPH Collection
CEPH	Centre d'Etude du Polymorphisme Humain
CHB	Han Chinese from Beijing, China Collection
CNV	copy number variant
COMT	catechol-O-methyltransferase
CYP	cytochrome P450
DBD	DNA binding domain
DMEs	drug metabolism enzymes
DNA	deoxyribonucleic acid
EGFR	epidermal growth factor receptor
eQTL	expression quantitative trait loci
ERK	extracellular signal regulated kinase
FDA	US Food and Drug Administration
FDR	false discovery rate
FMO	flavin containing monooxygenase
G6PD	glucose-6-phosphate dehydrogenase
GG	Illumina GoldenGate
GGCX	gamma glutamyl carboxylase
GPX	glutathione peroxidase
GST	glutathione-S-transferase
GWAS	genome wide association study
HWE	Hardy-Weinberg equilibrium

HNMT	histamine N-methyltransferase
HUGO	Human Genome Organization
IKP	Institut für klinische Pharmakologie
INR	International Normalized Ratio
ISI	International Sensitivity Index
isPCR	in-silico PCR
JPT	Japanese from Tokyo, Japan collection
KRAS	Kirsten rat sarcoma viral oncogene homolog
LBD	ligand-binding domain
LD	linkage disequilibrium
LE	linkage equilibrium
LSO	locus specif oligo
MAF	minor allele frequency
MAO	monoamine oxidase
MAPK	mitogen activated protein kinase
MDR	multidrug resistance
MPO	myeloperoxidase
MRP	multidrug resistance-associated proteins
NAPQI	N-acetyl-p-benzo-quinone imine
NAT	arylamine N-acetyltransferase
NCBI	National Center for Biotechnology Information
NGS	next-generation sequencing
Nrf	nuclear factor-erythoroid 2 p45-related factor
OAT	organic anion transporter
OATP	organic anion-transporting polypeptide
OCT	organic cation transporter
OP	Optimus Primer
OPA	oligonucleotide pool array
PAPS	3’-phosphoadenosine 5’-phosphosulfate
PCA	principal component analysis
PCR	polymerase chain reaction
PD	pharmacodynamics
PGP	P-glycoprotein
PHGS	prostaglandin H synthase
PI3K	phosphatidylinositol 3-kinase
PK	pharmacokinetics
PON	paraoxonase
PPAR	peroxisome proliferator activated receptor
PT	prothrombin time
PXR	pregnane x receptor
QC	quality control
RXR	the retinoid x receptors
SAM	S-adenosyl methionine
SNP	single nucleotide polymorphism
SLC	solute carrier
SULT	sulfotransferase

TMD	trans-membrane domains
TPMT	thiopurine S-methyltransferase
UDP	uridine diphosphate
UGT	UDP-glucuronosyltransferases
VKOR	vitamin epoxide reductase
XO	xanthine oxidase
YRI	Yoruba people of Ibadan, Nigeria Collection

Dedication and Acknowledgements

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1 Introduction

The work presented in this thesis details the development, optimization, validation and utilization of a pharmacogenomic drug metabolism genotyping panel and its application in research and clinical settings to identify pharmacogenomic biomarkers for many different classes of therapeutic agents. Specifically, this thesis will outline the development of a broad based drug metabolism genotyping panel that has extensive applicability in the realm of pharmaceutical drug development and personalized medicine. This tool can be used for basic research, for investigating variability in drug response phenotypes, as well as for screening patients in clinical trials during the drug development process. The Introduction summarizes the rationale for the project and delineates the primary objectives of the work. Additional sections of the Introduction have been devoted to providing relevant background information in the areas of pharmacology, genetics and genomic technology that are pertinent to the project.

1.1 Project Rationale

There is a current paradox in drug development. Drugs are developed in populations but are used to treat individuals. The large population-based clinical trials targeted to address public health concerns used by pharmaceutical companies are designed to assess the efficacy and safety of drugs in the greater public but do not presently take into account individual patient variability that can have drastic effects on dosing, efficacy and adverse reactions. This variability is the result of different environment influences (age, sex, weight, disease status, environmental exposures, other concomitant medications, etc.) but is also notably influenced by genetic variability. By incorporating genetics into the drug development pipeline, information about individual patient variability becomes part of the equation and leads to a greater understanding of

the observed variability in response and efficacy that is seen for different pharmaceutical therapies[1]. Additionally, our improved understanding of genetic variability in ADME genes has the potential to improve the use of many drugs already on the market. Presently, genetic variation has already been used to improve dosing regimens for drugs such as warfarin and as guidance for the optimal use of drugs such as clopidogrel[2,3].

There are two areas of pharmacology where genetics play a role in drug response, pharmacodynamics (PD) and pharmacokinetics (PK). PD involves the interaction of the drug with its target(s) and is directly responsible for the dose dependent pharmacologic response to the drug[4]. PK, determines the amount of the active drug that is available to interact with its target and for how long[5]. While the mechanisms of action (PD) of various drugs may differ, most drugs are metabolized by the products of a well-known, finite set of genes and pathways that influence their PK responses. Pharmacokinetic processes can be classified into four categories: Absorption, Distribution, Metabolism and Excretion (ADME). A genetic study involving a particular drug is not complete unless both PK and PD pathways are investigated; however an investigation of common ADME genetic pathways would be useful across many different studies or clinical trials involving any medication, while PD pathways are only specific to and useful for a given drug or treatment class.

In this thesis I will describe the development and validation of an ADME genotyping assay, including the selection of ADME content, and the testing of samples in research and clinical trials. Furthermore, I will also demonstrate the utility of the assay, and the added benefit it has over commercially available technologies in pharmacogenomic research. Finally, I will describe how the tools developed over the course of this project will have ongoing utility even as genomic technologies continue to evolve.

1.2 Objectives

The objectives of this project have been organized in four parts as outlined below:

1) ADME content selection
    a) Select genes with demonstrated involvement in drug metabolism
    b) Select markers with known and putative functional implications
    c) Standardize the description of ADME content.
    d) Select markers for maximum coverage of each gene up to space limitations of technology
2) Assay Development
    a) Select technology platform with greatest promise for success
    b) Understand nuances and limitations of technology
    c) Identify areas of genomic interference that reduce assay functionality
    d) Develop and design technological strategies to overcome limitations in technology and areas genomic interference
    e) Test and optimize design strategies
3) Assay Validation
    a) Test samples of known genotype to validate accuracy
    b) Test samples across additional technology platforms to validate technology
4) Assay Utilization
    a) Apply assay to research projects to demonstrate usefulness
    b) Demonstrate added value of assay compared to commercially available assays.

1.3 Pharmacokinetics

In order to make our drug metabolism genotyping assay as broadly applicable across as many therapeutic areas of medicine as possible, a thorough understanding of the pharmacokinetic pathways that are shared by many drugs was needed. By evaluating and better understanding these biochemical pathways, it is possible to hypothesize how genetic variation may disrupt the normal processes and lead to variation in the amount of active drug that reaches its site of action and therefore provide for the possibility to better predict what effects this variation might have on a patient's drug response. Below is a detailed overview of biotransformation pathways and the member enzymes that can influence drug response. The genes that encode these enzymes will comprise the majority of the content for the drug metabolism genotyping panel.

Pharmacokinetics (PK) refers to the biological processes that act upon drugs in the body. It involves Absorption, Distribution, Metabolism and Excretion (ADME). These processes are responsible for the length of time between administration and response, and for the duration and magnitude of this response. PK is dynamic, since the ADME processes all occur simultaneously on a given drug compound, and thus rates change over time. Several parameters can be measured and calculated to understand the PK of a given drug in a patient at a given time. Table 1 lists the common parameters that are used to characterize the PK of medical compounds.

Table 1: Common Pharmacokinetic Parameters

Category	Parameter	Formula
Absorption	Absorption rate constant	Rate of drug absorption ÷ Amount of drug remaining to be absorbed
	Bioavailability	Amount of drug absorbed ÷ Drug dose
Distribution	Apparent volume of distribution	Amount of drug in body ÷ Plasma drug concentration
	Unbound fraction	Plasma concentration of unbound drug ÷ Plasma drug concentration

Elimination	Rate of elimination	Renal excretion + Extrarenal (usually metabolic) elimination
	Clearance	Rate of drug elimination ÷ Plasma drug concentration
	Renal clearance	Rate of renal excretion of drug ÷ Plasma drug concentration
	Metabolic clearance	Rate of drug metabolism ÷ Plasma drug concentration
	Fraction excreted unchanged	Rate of renal excretion of drug ÷ Rate of drug elimination
	Elimination rate constant	Rate of drug elimination ÷ Amount of drug in body
		Clearance ÷ Volume of distribution
	Biologic half-life	0.693 ÷ Elimination rate constant

Source: *Kopacek, K.B.(2007)*[5]

The PK of a drug depends on a patient's phenotypic characteristics (such as age, weight and disease status) as well as the chemical properties of the drug (such as half-life and solubility). Variability in any of these factors can have profound effects on drug response. Many of these characteristics are well understood and are taken into account when determining the dose, dosing schedule and route of administration for a given medication; however, they are not sufficient to predict or explain a patient's personal drug response or toxicity.

1.3.1 Absorption

Absorption is the process by which the unchanged drug passes from the site of administration to the systemic blood circulation[6]. Absorption can be best characterized by the bioavailability parameter, which is the percent of administered dose that reaches the circulation without being modified[7]. There are several obstacles that can lower the bioavailabiliy of a drug. The administered drug must: be in the correct form (solid dosage forms must be dissolved), resist degradation, cross the physical barriers that separate the bloodstream from the site of administration, and for an orally delivered drug, it must resist the first-pass effect where drugs can be metabolized or cleared by the liver once they have entered the portal vein from the GI tract[7]. Drugs that are administered directly into the blood stream, intravenously, have a

bioavailability of nearly 100% and do not need to cross biological membranes versus those that are administered by other means, such as: orally, intramuscularly, transdermally, inhaled, sublingually, or subcutaneously that will have to pass through membrane barriers. Molecules may cross these membranes by three mechanisms: passive diffusion, facilitated transport, and endocytosis; the last of which plays little to no role in drug absorption[7].

Membrane permeability is a major determinant of how well a drug is absorbed. Biological membranes are fluid structures composed of phospholipids and proteins in varying proportions[8]. The degree of fluidity, and therefore permeability, depends on the type of phospholipids. Short chained unsaturated phospholipids are found in fluid membranes while long chained saturated phospholipids are found in rigid membranes[8]. Additionally, the nature of membrane proteins can also affect permeability. While peripherally bound proteins play little to no role in permeability, integral proteins that span the membrane can function as transporters while ion channels can alter the permeability. The permeability of the membrane will differ by tissue type and therefore, the site of administration will also influence the absorption. For example, there is lower impedance to absorption by the capillary wall between interstitial fluid and the bloodstream (subcutaneous and intramuscular administration) than the epithelial lining of the GI tract and the bloodstream (oral administration) [7].

The chemical properties of the drug will also affect how well it can be absorbed. Small molecules (< 500-700 Daltons) are able to passively cross cellular membrane with relative ease, however as molecular size increases, the solubility properties of the drug play the most significant role in its absorption[9]. Lipophilic drugs will readily dissolve in the lipid membrane, while hydrophilic drugs will dissolve in the water that carries them throughout the body. Lipid soluble molecules are able to cross the membrane down the concentration gradient. Since the

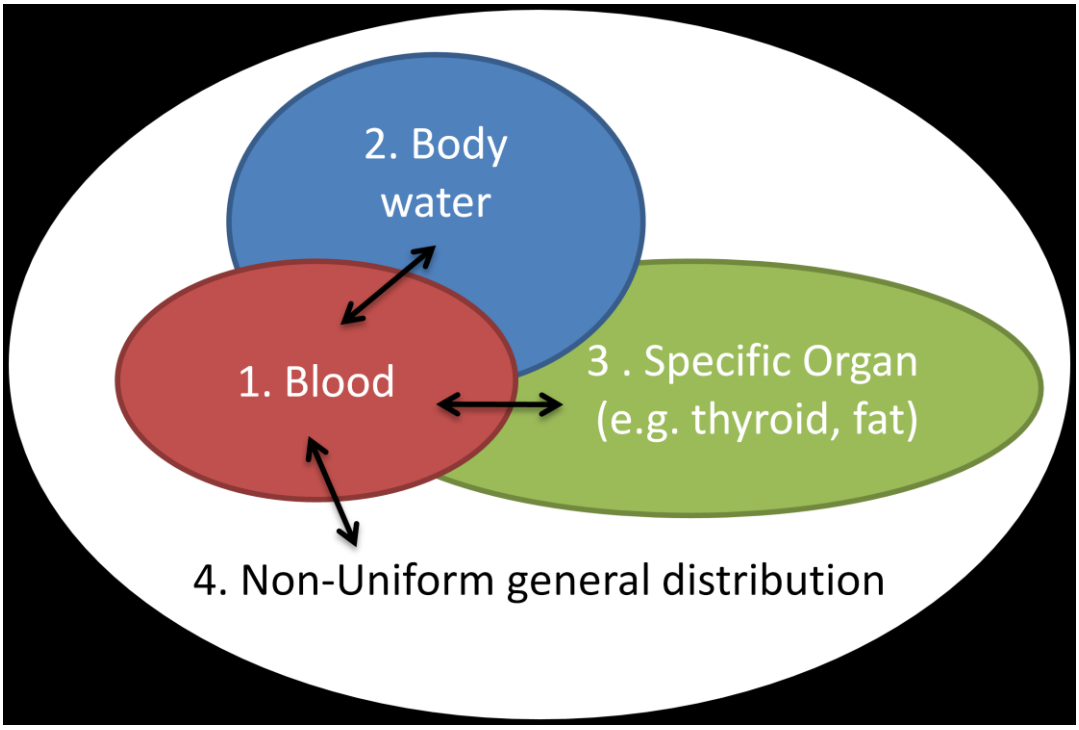
circulating concentration is negligible, due to the large volume and constant flow, the rate of absorption for lipid soluble molecules is directly proportional to the concentration on the donor side of the membrane [9]. In addition to solubility, molecular size, degree of ionization and the number of hydrogen bonds will also affect the absorption of drugs[8]. The rate of diffusion is inversely proportional to the size of the molecule, the ratio of ionized to unionized forms and the number of hydrogen bonds[8].

While traditional thinking states that passive diffusion plays the largest role in drug absorption, it is now becoming clear that carrier mediated transport, both passive (energy independent down a concentration gradient) and active (energy dependant against a concentration gradient), plays a very important role[10]. Influx and efflux transporters are present in all cell types and tissues. They are able to bind reversibly to drug molecules and carry them across biological membranes. The overall intracellular concentration at equilibrium of a drug will be the result of the effects of both passive diffusion and transporters taking into account both influx and efflux pathways.

1.3.2 Distribution

Distribution describes the process by which drugs move within the body from the blood to organs, tissues and back. The distribution of a drug to its site of action is the link between pharmacokinetics and pharmacologic activity of the drug[8]. There are four observable patterns of distribution that can influence the pharmacologic response of a drug (see Figure 1: Patterns of Drug Distribution)[7].

Figure 1: Patterns of Drug Distribution



Adapted from *Benedetti et al. 2009*[4]

1. A drug may remain for the most part in the bloodstream; this can occur when the drug is largely bound to plasma proteins that inhibit its transport across plasma membranes. 2. A drug can be equally distributed throughout all of the body's water; this usually occurs to small water soluble molecules. 3. A drug may be concentrated in a specific organ or tissue such as the thyroid or adipose tissue. 4. A drug may show a non-uniform distribution pattern consisting of a combination of the previous three distribution patterns.

The pharmacokinetic parameter that best represents distribution is the volume of distribution which is calculated by dividing the amount of administered drug by the measured plasma concentration. A volume of distribution greater than the total body water indicates that the drug has been concentrated into tissues whereas a volume of distribution smaller than total body water indicates that the drug remains in the plasma[7]. Warfarin, an acidic drug, for example, remains largely protein bound and therefore has a small apparent volume of distribution; while a

basic drug such as amphetamine will be taken up into tissue readily and has a very large apparent volume of distribution[5].

The drug characteristics that affect distribution are the same as those that influence absorption; size, solubility, and ionization status. Lipid soluble molecules may end up sequestered in fat tissue whereas large water soluble molecules may remain in circulation. This also means that a person's body composition can influence drug distribution. A person with a higher percent body fat will have a greater distribution volume than a leaner person[11].

Other factors that influence drug distribution include the blood perfusion of a given tissue which increases the availability of the drug and binding of drugs to plasma proteins, the most common being albumin, as only unbound drugs can cross membranes[7]. Finally, as in absorption, membrane permeability plays a major role in distribution as drugs must cross the barriers separating the circulatory system from the various tissues and cell types. This may occur by simple diffusion, passive transport, or active transport. A drug cannot exert its effects unless it reaches its site of action, therefore variability that influences the distribution pattern of the drug can have profound effects on response.

1.3.2.1 The Blood Brain Barrier

A unique barrier that drugs whose pharmacological action targets the brain must cross is the blood brain barrier (BBB). There are three components to the BBB that make it difficult for drugs to cross, especially polar water soluble compounds. First, the cerebral endothelial cells form tight junctions with each other effectively sealing off the paracellular space[12,13]. Second, several membrane transporters, such as P-glycoprotein and members of the multidrug resistance protein family, are present in cerebral endothelial cells that regulate the transport between the

brain and the blood and serve as a protective efflux system to potentially harmful substances. Finally, the presence of metabolic enzymes, such as monoamine oxidase (MAO) and several cytochrome P450 enzymes, add additional metabolic activity to assist with the detoxification and elimination of compounds that attempt to cross the BBB[9, 10].

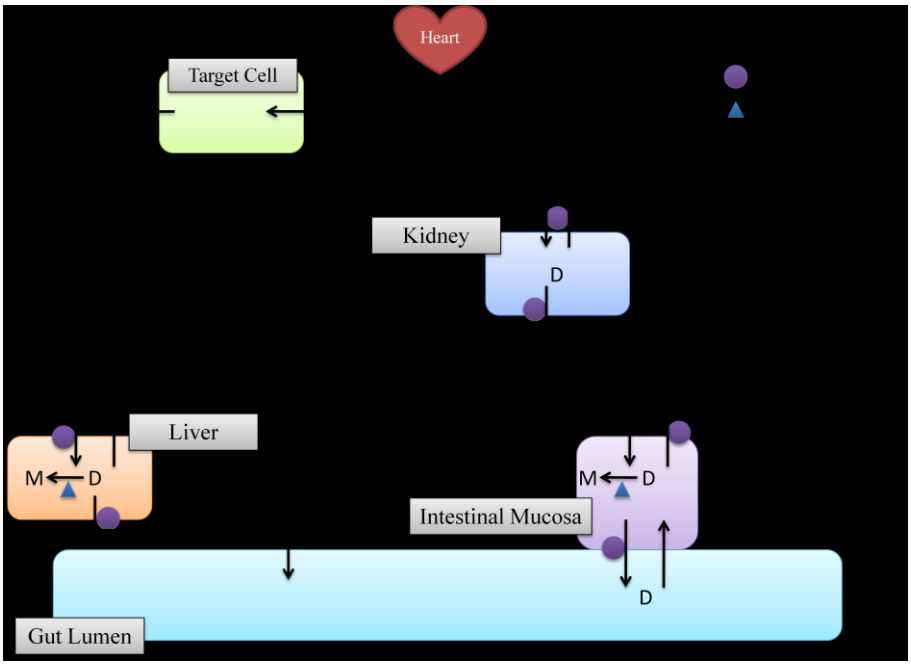
1.3.3 Metabolism

Drug metabolism describes the modifications involved in the biotransformation of drugs. It can be thought of as the body's defense mechanism against xenobiotics. These defense mechanisms have been evolving for billions of years[14]. From an evolutionary standpoint, it is believed that through natural selection, external pressures, such as exposures to toxic compounds; either accidentally (such as toxic compounds in the diet) or on purpose (such as the administration of drugs), have led to the selection of novel isoforms of genes with endogenous functions that provide a selective advantage by helping to regulate and limit the exposure and or accumulation of xenobiotic agents[15–17].

Overall drug metabolism renders target molecules more water soluble and thus easier to excrete. There are four fates to the products of drug metabolism: a metabolite may have decreased activity compared to the administered drug, a metabolite may be more active than the parent compound; which is the case for a prodrug where the metabolite not the parent compound is responsible for the pharmacologic activity; a metabolite may be toxic, or a metabolite may have a physiological effect unrelated to the parent drug. Most compounds are metabolized through a number of pathways, either in parallel or in sequence, with each pathway having different outcomes with regard to drug response. The relative amount of each metabolite formed differs between tissues and among individuals. It depends on physiological conditions such as availability of enzyme, cofactors, modulators of cellular biochemistry, and transport of substrates

in and out of cells[18]. Metabolic enzymes are not uniformly distributed throughout the body and can even be induced or repressed by various stimuli (see sections 1.3.3.1, 1.3.3.2 and 1.3.6 below). Also, with a finite number of active sites for any given enzyme in a given tissue, it is possible for these sites to become saturated with the drug in question or with other molecules, both endogenous and exogenous, that are competing for the active site. Additionally, demographic characteristics such as age, BMI, disease state and environmental exposures such concomitant medications can also influence drug metabolism[18].

Figure 2: Movement of a Drug in the Body



Adapted from: *Thummel KE. 2007*

Metabolic reactions occur though chemical modifications that take place under normal physiological conditions (i.e. neutral pH, 37°C, atmospheric pressure etc.). In order to increase the rate of these reactions, enzymes are required. Enzymes facilitate these reactions in two ways, approximation and catalysis. Approximation involves the bringing together of two substrates in the proper three-dimensional orientation for the reaction to proceed. Catalysis involves the

reduction in activation energy required for the reaction to proceed. The enzyme interaction provides an alternative reaction route by stabilizing intermediates and thus increases the number of substrates with enough energy to pass through the transition state and form the product. The binding between enzymes and their substrates occurs mainly through non-covalent interactions.

The majority of metabolic reactions, for both endogenous chemicals such as cholesterol or steroid hormones and xenobiotics, occur in the liver. Compounds that are absorbed via the small intestine into the portal vein will pass through the liver prior to any other organ or tissue. This is known as the first-pass effect as many drugs are extensively metabolized or cleared from circulation prior to reaching any additional site in the body. Even if a portion of a drug makes it past the liver, or is administered in such a way to bypass the first pass effect (by IV for example), the subsequent passes through the liver as part of normal circulation will result in further metabolism of the drug (see Figure 2: Movement of a Drug in the Body)[19]. Other organs that have been shown to contain drug metabolizing enzymes to a smaller degree include the small intestine, the lungs, the nasal mucosa and kidneys.

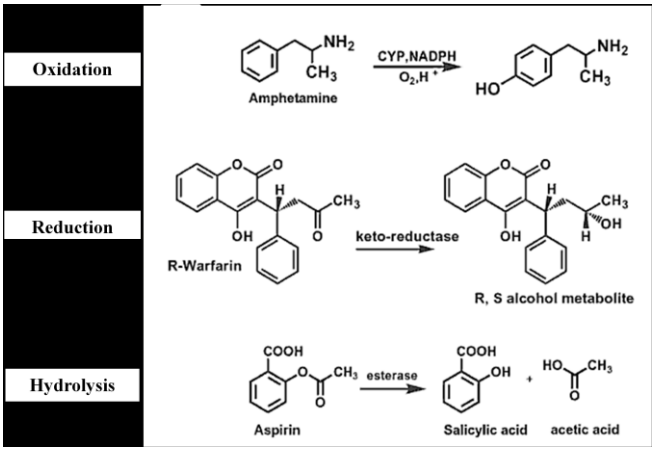
Drug metabolism enzymes (DMEs) fall into two categories. Phase 1 DMEs responsible for the functionalization reactions that either introduce or unmask polar functional groups making molecules more water soluble; and phase 2 DMEs responsible for conjugation reactions that attach hydrophilic chemical moieties to drugs.

1.3.3.1 Phase I Drug Metabolism Enzymes

Phase I drug metabolism describes the oxidation, reduction and hydrolysis reactions that occur to xenobiotics (see Figure 3: Phase I Drug Metabolism Reactions)[15] . These reactions result in either the introduction of new polar groups (oxidation), the modification of existing functional groups (reduction) or the unmasking of existing polar functional groups (hydrolysis)[15].

Often, the product of phase I drug metabolism will not be sufficiently polar to be excreted and must undergo further metabolism by either additional phase I or phase II enzymes.

Figure 3: Phase I Drug Metabolism Reactions

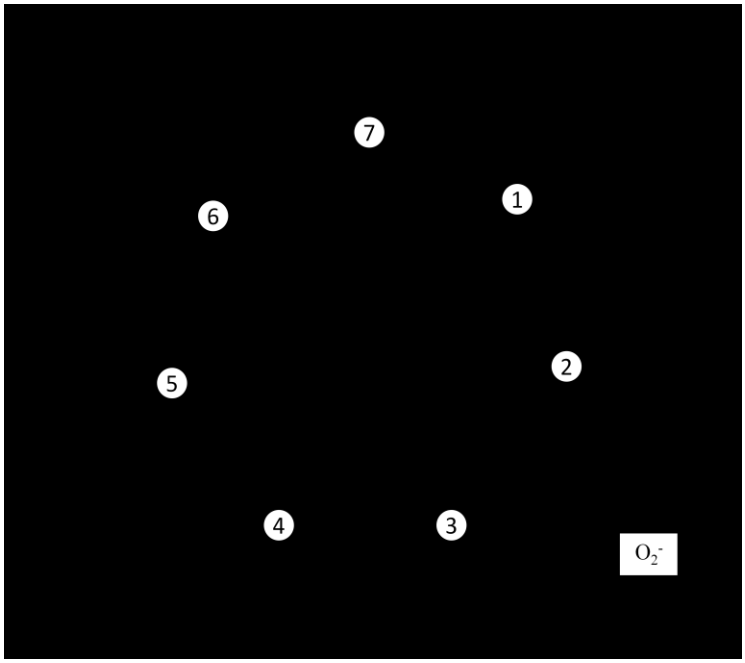


Source: Nassar AF, Hollenberg PF, Scatina J. *Drug Metabolism Handbook*. John Wiley and Sons; 2009.

1.3.3.1.1 Cytochrome P450 Enzymes

The most abundant phase I DMEs are the cytochrome P450 (CYP) enzymes, a superfamily of haeme containing mono-oxygenases divided into sub-families based on amino acid sequence homology. The CYP superfamily contains 57 functional genes and an additional 58 pseudogenes that play a role in endogenous compound metabolism, however only members of the 1,2 and 3 families play significant roles in drug metabolism[20]. Five CYPs in particular; 1A2, 2C9, 2C19, 2D6 and 3A4, are involved in about 95% of CYP-mediated metabolism and metabolize about 80% of drugs currently on the market[7,20]. The most common CYP 450 reaction is the mono-oxidation of a substrate (See Figure 4).

Figure 4: P450 Monooxygenase Mechanism



R = any side chain

The active site of all CYP enzymes contains a haeme moiety. When a substrate binds to the active site (1), there is a change in the electronic state of the haeme that favors reduction from Fe^{2+} to Fe^{3+} with NADH or NADPH as the electron donor (2). This is followed by the binding of oxygen to the haeme (3). This state is unstable and a decoupling reaction can occur resulting in the release of a superoxide radical. If however, a second electron is transferred to the moiety the reaction can continue (4). The peroxo grouped formed with the addition of the second electron is rapidly protonated from the surrounding solvent twice, releasing a water molecule and leaving an activated oxygen atom (5) that is able to react with the substrate molecule (6) that results in a hydroxylated substrate and the enzyme in its initial state (7)[17].

1.3.3.1.2 Non CYP Oxidative Metabolism

In addition to CYP enzymes which are the most prevalent, the products of several other gene families also contribute to oxidative drug metabolism, these include: flavin-containing

monooxygenases (FMO), monoamine oxidases (MAO), alcohol (ADH) and aldehyde dehydrogenases (ALDH), and some peroxidases (see Table 2).

Table 2: Phase 1 Oxidation Enzymes

Enzyme Family	Oxidative Reaction
Cytochrom P450	$RH+O_2+2H^+ +2e^- \rightarrow ROH+H_2O$
Flavin Containing Monooxygenase	$RNH+O_2+2H^+ +2e^- \rightarrow RNOH+H_2O$
Monamine Oxidase	$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$
Alcohol dehydrogenase	$ROH + NAD^+ \rightarrow RO + NADH + H^+$
Aldehyde dehydrogenase	$RCHO + NAD^+ + H_2O \rightarrow RCOOH + NADH + H^+$
Peroxidase	$ROOR' + 2H^+ + 2e^- \rightarrow ROH + 2X' + R'OH$

There are five members in the FMO family (FMO1-5) with FMO3 being the most studied due to its association with trimethylaminuria[21]. FMO enzymes oxygenate substrates that contain soft-nucleophiles usually sulfur or nitrogen[22]. The biological function of FMO is poorly understood, however many FMOs share substrate specificity with many CYPs yet often result in different products. Xenobiotics that are metabolized by FMO enzymes include ketoconazole, and tamoxifen[23,24]. Unlike CYP enzymes, FMOs do not require a reductase to transfer electrons from NAD(P)H and do not contain a haeme moiety, additionally FMO enzymes are not readily induced or inhibited and therefore are not as prone to drug-drug interactions [21].

There are two members of the MAO family (A and B) that catalyze oxidative deamination of monoamines; most notably monoamine neurotransmitters in neurons and astroglia[25]. However, both MAO-A and B are also found outside the central nervous system and can play a role in the catabolism of ingested monoamines[25]. Some of the xenobiotics known to be metabolized by either MAO A or B include β-blockers such as metoprolol and propranolol and serotonin reuptake inhibitors such as sertraline and citalopram[24].

Dehydrogenase enzymes utilize a hydride (H^-) acceptor, usually $NAD(P)^+$ to oxidize a substrate. ADH enzymes catalyze the conversion of alcohols to aldehydes and can be grouped

into five classes in humans. ADH enzymes exists as a either a homo or hetero-dimer and there are at least 7 different genes that encode subunits (isoenzymes) of the enzyme[24]. Class 1 ADH is the primary hepatic form, responsible for the conversion of ethanol to acetylaldehyde, and is made of three subunits encoded by the genes ADH1A, ADH1B and ADH1C[24]. ADH has also been implicated as the primary metabolism pathway for several pharmaceuticals (which are also alcohols) such as abacavir and ethambutol[26–28].

There are 17 genes that encode for members of the ALDH super-family (grouped into 10 families and 13 subfamilies based on amino acid sequence homology) that catalyze the conversion of aldehydes into carboxylic acids[24]. ALDH enzymes have several very important endogenous functions such as the conversion of 10-formyl-tetrahydrofolate to tetrahydrofolate or the oxidation of retinaldehyde to retinoic acid and inborn errors in aldehyde metabolism are generally associated with neurological abnormalities[24,29,30]. The role of ALDH in xenobiotic metabolism has also been demonstrated; in addition to the conversion of acetylaldehyde to acetic acid in ethanol metabolism, ALDH is also responsible for the inactivation of the aminoaldehyde form of 4-hydroxycyclophosphamide, the active form of cyclophosphamide[31].

Peroxidases are enzymes that have the unique ability to catalyze the single electron oxidation of substrates. The endogenous role of peroxidases such as glutathione peroxidase (GPX) is the detoxification of hydrogen peroxide, however, the formation of oxidized glutathione has been observed after the administration of certain drugs which would indicate that single electron oxidation is taking place resulting in radical formation and oxidized glutathione[32,33]. In addition to GPX, peroxidases known to be involved in drug metabolism include: myeloperoxidase (MPO), which is able to oxidize chlorine containing compounds such

as clozapine; and prostaglandin H synthase (PHGS), involved in the oxidation of paracetamol[24,34,35].

1.3.3.1.3 Phase I Reduction Reactions

While oxidative processes account for the majority of phase I drug metabolism, some reductive reactions have been identified and may play a significant role in drug metabolism. Reduction reactions are carried out mainly by members of the CYP family with NADPH as a reductive cofactor. Several drugs are known to undergo reductive metabolism, such as the antifertility drugs norgesterl and norethindrone, due to the presence of the reduced metabolite in urine, however the exact mechanism is not always known[36]. In other cases, the reductive mechanism is known, as is the case with clonazapam reduction by CYP3A4[37].

1.3.3.1.4 Phase I Hydrolysis Reactions

Hydrolytic reactions are carried out by esterases that split esters into an acid and an alcohol. Esterases important to drug metabolism are carboxylesterases (encoded by members of the CES gene family), arylesterases (encoded my members of the PON gene family), and cholinesterases (encoded by the genes ACHE and BCHE). Drugs that undergo hydrolytic metabolism include procaine, and phensuximide[38,39].

1.3.3.2 Phase II Drug Metabolism Enzymes

Phase II drug metabolism reactions, or conjugation reactions, involve the transfer of small polar endogenous molecules to drugs. The new chemical moiety is usually activated by a coenzyme then transferred to a functional group, typically alcohols, amines, carboxylic acids and thiols, on the parent drug itself or to one introduced in the product of phase I metabolism (Table 3). The resulting metabolites are more water soluble and thus easier to excrete and/or have

reduced activity. Phase II reactions that play the biggest role in drug metabolism include: glucuronidation, glutathione conjugation, acetylation, sulfation, methylation and amino acid conjugation[15].

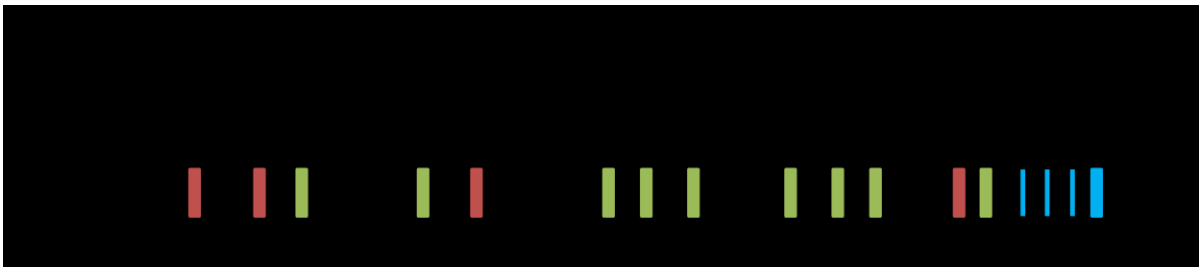
Table 3: Phase II Drug Metabolism Reactions

Functional Group	Reaction	Source	Known Substrates
-COOH	Amino acid conjugation	glycine or glutamine	benzoic acid
-COOH	Glucuronidation	uridine 5'-diphospho-glucuronic acid (UDPGA)	bilirubin, irinotecan
$-NH_2$, -OH, -SH	Methylation	S-adenosyl methionine (SAM)	Nicotine, azathioprine
$-NH_2$, -OH	Sulfonation	3'-phosphoadenosine-5'-phosphosulfate (PAPS)	minoxidil
$-NH_2$, -OH	Acetylation	acetyl-CoA	isoniazid
-Cl, -Br, -I, -F, $-NO_2$, -C=C-	Glutathione conjugatoin	reduced glutathione	N-acetyl-p-benzoquinone imine, thiotepa

1.3.3.2.1 Glucuronidation

Glucuronidation is catalyzed by uridine diphosphate (UDP)-glucuronosyltransferases (UGT). UGT enzymes can be grouped into two families based on the gene location. The UGT1A locus, located on chromosome 2, consists of 9 genes and 4 pseudogenes in roughly a 200 kb region. An interesting feature of the locus is that all 13 genes share the same exons 2-5 and have a unique first exon (see Figure 5). The common 245 amino acid sequence at the carboxyl terminus, encoded by exons 2-5, determines the interaction with the common substrate, UDP-glucuronic acid, while the amino terminal region encoded by the first exon determines substrate specificity[40].

Figure 5: UGT1A Locus



Members of the UGT1A family are responsible for the glucuronidation of a wide variety of endogenous compounds such as thyroxine, estrone and bilirubin. UGT1A1 is the most studied gene of the 1A locus having the greatest bilirubin conjugating activity [41]. Reduced function of UGT1A1 can lead to hyperbilirubinemic syndromes such as Gilbert's syndrome or more severe forms such as Crigler-Najjar syndrome[42]. UGT1A enzymes are also responsible for the glucuronidation of several xenobiotics. Of note is the chemo therapeutic irinotecan, where reduced function in UGT1A enzymes leads to reduced detoxification of 7-ethyl-10-hydroxycamptothecin, an active metabolite of the drug [43].

The UGT2 family is further divided into two subfamilies, UGT2A consisting of two genes (2A1 and 2A2) and UGT2B consisting of seven genes. The 9 genes that encode members of the UGT2 family are located on chromosome 4. Endogenously, UGT2 enzymes are responsible for the glucuronidation of many compounds such as hyodesoxycholic acid as well as various steroid hormones and fatty acids. Drugs that are metabolized by UGT2 enzymes include morphine (UGT2B7), alizarin (UGT2B17) and oxazepam (UGT2B15) [40,44–47].

1.3.3.2.2 Glutathione Conjugation

In addition to the antioxidant abilities of glutathione in the removal of reactive oxygen species; glutathione conjugation to endogenous and exogenous lipophilc compounds is an

important process in reducing toxicity and increasing excretion. Glutathione conjugation is carried out by glutathione-S- transferase (GST) enzymes. These enzymes can be divided into seven classes based on amino acid identity, alpha, kappa, mu, omega, pi, theta and microsomal. GSTs are highly expressed and account for up to 10% of cytosolic protein in some organs. GST enzymes occur as a either a homo or hetero dimers with both subunits being from the same class[48]. GSTs are highly inducible with over 100 endogenous and exogenous compounds found to increase GST expression; many of the compounds that induce GSTs are themselves substrates for the enzymes[49]. Genes that encode GSTs in humans with the greatest amount of inter-individual variation are members of the mu (GSTM1-5), pi (GSTP1), alpha (GSTA1-4) and theta (GSTT1-2) classes. Xenobiotics known to be metabolized by GST enzymes include N-acetyl-p-benzo-quinone imine (NAPQI); the toxic metabolite of acetaminophen, thiotepa, as well as other chemotherapeutic agents which has led to the hypothesis that GSTs play a role in tumor drug resistance[44,50–52].

1.3.3.2.3 Acetylation

The phase II acetylation reaction usually involves the transfer of an acetyl group (CH_3CO), from acetyl CoA, to a variety of nitrogen containing compounds (N-acetylation), however O-acetylation has also been observed[53,54]. Acetylation does not make the substrate more polar, rather it usually serves to either detoxify the substrate or to activate it[55]. There are two enzymes responsible for acetylation drug metabolism reactions, N-acetyltransferase 1 and 2 (NAT1 and 2). NAT enzymes are among the most studied ADME enzymes and there is a vast amount of molecular, structural and biochemical information pertaining to them[56]. The N-acetylation of drugs by NAT enzymes was one of the first polymorphic drug metabolism phenotypes observed over 50 years ago when it was recognized that there were large inter-

individual differences in the toxic side effects of the drug isoniazid[57,58]. In addition to isoniazid, other drugs such as sulfur-containing antirheumatic and anti-inflammatory drugs have side effect profiles that are related to NAT activity[55].

1.3.3.2.4 Sulfonation

Sulfonation, catalyzed by members of the sulfotransferase (SULT) gene family of enzymes, involves the transfer of a sulfonate (SO_3^-) group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to a hydroxyl or amino group of a substrate[59]. The sulfonation of endogenous and xenobiotics can serve to increase water solubility and thus excretion, which is the case for the drug acetminophen and the neurotransmitter dopamine. However, in some cases as is the case for the drug minoxidil, the sulfonation reaction is necessary for the drugs pharmacologic activity [60].

There are two types of SULT enzymes in humans, those that are bound to the golgi apparatus responsible predominantly for the sulfonation of peptides, proteins and lipids, and cytosolic SULTs that are responsible for the sulfonation of xenobiotics. There are 13 cytosolic SULTs that can be grouped into three families based on amino acid sequence identity: SULT1 (A1, A2, A3, A4, B1, C2, C4, E1), SULT2 (A1, B1_v1, B1_v2) and SULT4 (A1_v1 and A1_v2) [59]. Many SULT enzymes share substrate specificity with UGT enzymes. It has been proposed that the SULT system represents the low affinity high capacity pathway while the UGT system represents the high affinity low capacity pathway for these substrates [59].

1.3.3.2.5 Amino Acid Conjugation

Amino acid conjugation was the first conjugation reaction characterized in humans with the demonstration that benzoic acid was metabolized to hippuric acid[61,62]. Despite its early

discovery, amino acid conjugation is not as well characterized as other drug metabolism reactions. The reaction is catalyzed by acyl transferase enzymes and results in the formation of an amide bond between a carboxyl functional group and an amino acid. Glycine is the amino acid most often conjugated in humans[61]. Many drugs undergo phase I metabolism that results in the formation of a carboxyl group that can be conjugated to glycine. These include the antihistamines astemizole and brompheniramine[61].

1.3.3.2.6 Methylation

Methylation involves the transfer of a methyl group (-CH3) and is involved in the regulation of several biological processes. This reaction is catalyzed by several different methyltransferase enzymes throughout the body. The most common donor of endogenous methyl groups is S-adenosyl methionine (SAM). The NH2, OH and SH functional groups on xenobiotics and on endogenous molecules such as DNA, histones, and neurotransmitters have the potential to be methylated.

The methyltransferase enzyme with the greatest amount of evidence concerning its involvement in drug metabolism is thiopurine S-methyltransferase (TPMT) that catalyzes the S-methylation of aromatic and heterocyclic sulphydryl compounds such as azathioprine[63–65]. Other methyltansferases that have been proposed to be involved in drug metabolism include histamine N-methyltransferase (HNMT) and catechol-O-methyltransferase (COMT) with selective serotonin reuptake inhibitor response[66–68].

1.3.4 Excretion

Excretion is the process of removing a drug from the body and expelling it to the external environment. The drug can be excreted in the form of an unchanged molecule or as a metabolite.

The pharmacokinetic parameter used to quantify excretion is clearance; which is a measure of the volume of blood or plasma that has been irreversibly cleared of the drug per unit of time. A drug may undergo several routes of excretion; therefore total clearance is the sum of the individual excretion pathways. Since a drug or metabolite must cross biological membranes to exit the body, the same chemical and physical properties that influence absorption and distribution for a given drug also govern its excretion rates in excretory organs. Additionally, the same mechanisms, passive diffusion and facilitated transport, are involved in excretion. Excretion takes place primarily in two organs, the kidney and the liver. Renal excretion is controlled by three processes: Glomerular filtration in Bowman's capsule, which is a passive process; tubular secretion, which is predominantly an active process involving transporters; and tubular reabsorption, which can occur through both passive diffusion and active transport. In the liver, in addition to the presence of metabolism enzymes, excretion can occur in the form of bile. Biliary excretion occurs primarily via active transport, as small lipophilic molecules are reabsorbed in the bile duct. Conjugation reactions often facilitate biliary excretion as these metabolites are larger in size and are not as readily reabsorbed. It is possible for drugs that enter the gut through the biliary system to be reabsorbed. This is known as enterohepatic recycling.

1.3.5 Transporters

Transporters are membrane proteins that are able to transport both endogenous compounds and xenobiotics across biological membranes. Transporters are located throughout the body, anywhere that compounds need to cross membranes and are unable to do so by passive diffusion alone. Transporters are involved in drug absorption, in the intestinal lumen for example, in drug distribution, as part of cellular uptake or across the blood-brain barrier for example, and in drug elimination, in the liver and kidney for example[15]. Depending on the tissue

type, a given epithelial cell may have both influx and efflux transporters for the same compound, therefore it is the combined action of different transporter types that will determine the overall direction and scale of drug movement[69]. Additionally, there may be redundancy in certain tissues, where transporters with overlapping substrate specificities are often present together. From an evolutionary perspective this redundancy may be protective ensuring that toxic compounds are readily removed[69].

Transporters can be separated into two major classes: the solute carrier (SLC) family of proteins and the adenosine triphosphate-binding cassette (ABC) transporter superfamily. Both types of transport proteins are integral membrane proteins with trans-membrane domains (TMDs) joined by intra- and extra- cellular chains or loops of amino acids. The substrate binding site can be formed by the amino acid sequence of the intracellular loop in the case of efflux transporters (ABC and SLC members), the extracellular loop in the case of influx transporters (SLC members) or the TMD in the special circumstance of some ABC transporters[70].

1.3.5.1 Solute Carrier Proteins

The SLC family is the largest superfamily of transporters with over 300 genes presently known to encode SLC members[70]. SLC transporters are classified by their substrate specificity such as organic anions (OAT), organic cations (OCT), and organic anion polypeptides (OATP). Not all SLC transporters however, are involved in the transport of drugs. The most important SLC transporters involved in drug transport are the members of the SLCO1B, SLC15A, and SLC22A families[69] (see Table 4: Transporters important to Human Drug Transport).

SLC transporters use electro-chemical gradients as the driving force for transport[71]. They are generally co-transporters, transporting ions and solutes simultaneously in either the same or

opposite directions[70]. The Na^+ ion gradient is the most common source of energy in humans for SLC transporters; however other gradients include H^+, glutathione, HCO_3^- and α-ketoglutarate[70].

1.3.5.2 ATP-binding Cassette Transporters

Currently, 49 ABC transporters have been identified and grouped into 7 subfamilies (A through G). However only P-glycoprotein [ABCB1 also know as multidrug resistance 1 (MDR1)], several members of the multidrug resistance-associated proteins (MRP1-9 or ABCC1-9) and the breast cancer resistance protein (BCRP, ABCG2) have been shown to be efflux drug transporters[70,72] (see Table 4: Transporters important to Human Drug Transport). ABC transporters are highly expressed in important pharmacological barriers such as the blood-brain barrier (BBB), excretory sites such as the biliary canalicular membrane of hepatocytes and absorption barriers such as the membrane of intestinal cells[73]. ABC transport usually occurs against a concentration gradient and requires intracellular production of ATP as an energy source[7]. ABC transporters contain a cytoplasmic ATP binding domain that binds and hydrolyses ATP and drives drug transport[73].

Table 4: Transporters important to Human Drug Transport

Gene name	Transporter Name (synonym)	Tissue distribution (Important to PK)	Cell localization
ABC:			
ABCB1	Pgp (MDR1)	Blood–brain barrier, Liver, Intestine, Kidney,	Apical
ABCB11	BSEP (SPGP)	Liver	Apical
ABCC1	MRP1 (MRP)	Lung, Kidney, Peripheral Blood	Basolateral , apica
ABCC2	MRP2 (cMOAT)	Blood–brain barrier, Liver, Intestine, Kidney,	Apical
ABCC3	MRP3 (MOAT-D)	Intestine, Liver, Kidney	Basolateral
ABCC4	MRP4 (MOAT-B)	Kidney, Lung	Apical, basolateral
ABCC5	MRP5 (ABC11)	Liver, Brain	Apical,

			basolateral
ABCG2	BCRP (MXR)	Blood–brain barrier, Liver, Intestine	Apical
SLC:			
SLCO1B1	OATP1B1 (OATP-C)	Liver	Basolateral
SLCO1B3	OATP1B3 (OATP8)	Liver	Basolateral
SLC15A1	PEPT1 (HPEPT1)	Intestine	Apical
SLC15A2	PEPT2	Intestine	Apical
SLC22A1	OCT1	Liver, kidney, intestine	Basolateral
SLC22A2	OCT2	Liver, kidney, intestine	Basolateral
SLC22A6	OAT1 (NKT)	Liver, Kidney	Basolateral

Source: *Sugiura, Kato, and Tsuji 2006; Szakács et al*. 2008*; Klaassen and Aleksunes* 2010

1.3.6 Modifiers of Drug Metabolism

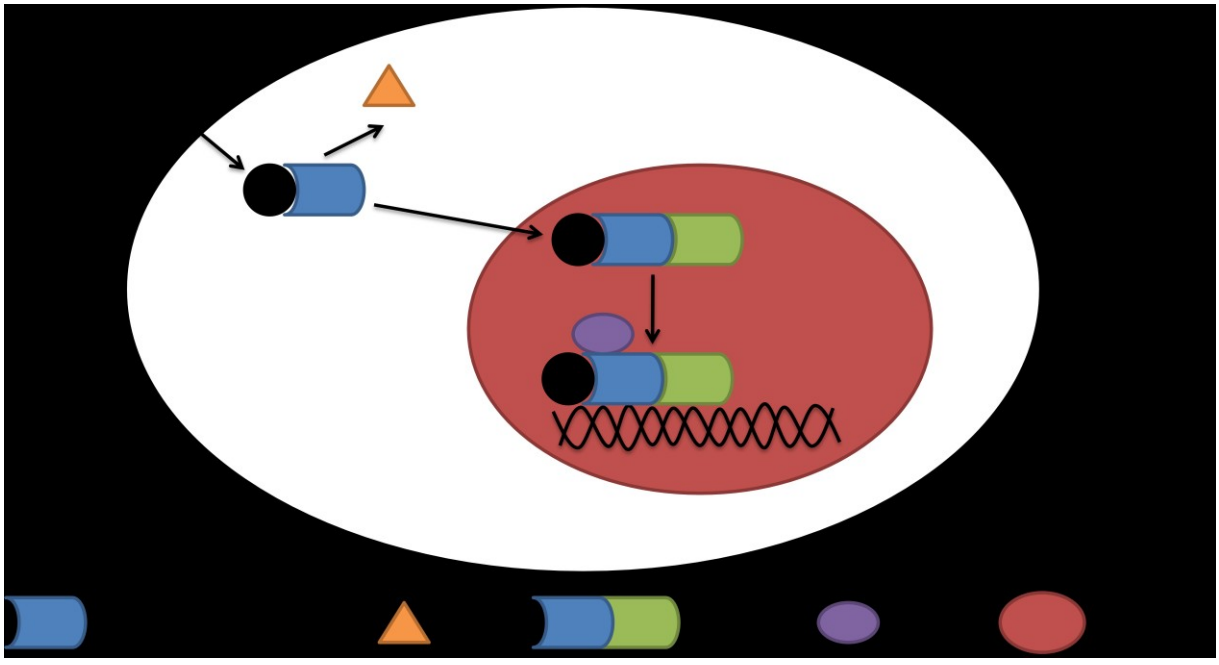
In addition to drug metabolism pathways themselves, the regulation of drug metabolism genes also plays a critical role in determining ones pharmacokinetic profile. Many DME and transporter genes share common mechanisms of transcriptional activation through various nuclear receptor signaling cascades. These include the aryl hydrocarbon receptor (AhR), orphan nuclear receptors and nuclear factor-erythoroid 2 p45-related factor 2 (Nrf2). There is a significant overlap in regulatory targets for most of these transcription factors. In fact, this overlap can account for some drug-drug interactions as the administration of one drug may upregulate the metabolism or excretion of another. Additionally, autoinduction, the process where a therapeutic agent triggers the increase in its own clearance, is also a common phenomenon and is the primary mechanism of pharmacokinetically mediated tolerance[74].

1.3.6.1 Nuclear Receptors

The expression of drug metabolism enzyme genes and drug transporter genes are regulated via similar pathways; notably by members of the nuclear receptor superfamily. There are 49 known members of the nuclear receptor superfamily and common to all members are three major protein domains categorized based on function 1) an activation function 1 domain

(AF-1) at the amino terminus responsible for ligand-independent activation through interaction with additional transcription factors. 2) A DNA binding domain (DBD) centrally located in the protein that interacts with DNA response elements. 3) A ligand-binding domain (LBD) located at the carboxy-terminus[75]. Upon binding of a drug or other ligand to the LBD in the cytoplasm, the receptor is dissociated from any corepressors to which it may be binding, usually a histone deacetylase and translocates to the nucleus to act as a transcription factor. Once inside the nucleus, the receptor forms either a homo or hetero complex and binds via the DBD to the appropriate DNA response elements in regulatory regions of target genes. The presences of the protein complex in the regulatory domain subsequently impact/regulate cognate gene transcription by interacting with the cellular transcriptional machinery (See Figure 6)[75].

Figure 6: Nuclear Receptor Activation



Adapted from Urquhart et al 2007

When a ligand binds to a nuclear receptor, it is transported to the nucleus where it can serve as a transcription factor and initiate transcription.

Generally, when a cell is exposed to a drug, the xenobiotic substrate will interact with the nuclear receptors present in the cytoplasm triggering a transcription factor cascade into the nucleus that leads to increased expression of the enzymes and transporters that will reduce the exposure [76,77]. The most notable nuclear receptors involved in drug metabolism are the: Aryl hydrocarbon receptor (AhR), the orphan nuclear receptors, and nuclear factor-erythoroid 2 p45-related factor 2 (Nrf2).

1.3.6.2 Aryl hydrocarbon receptor

AhR is a highly polymorphic transcription factor that forms a dimer with the aryl hydrocarbon nuclear translocator (Arnt), which is not required for nuclear transport, however when bound together it causes a conformational change that results in greater recognition of enhancer sequences and thus activation of target genes[78]. AhR, when bound by polycyclic aromatic hydrocarbons such as dioxin, increases the expression of the CYP1 family members and has also been shown to up regulate UGT1A1, UGT1A6 and GSTA1 [79,80].

1.3.6.3 Orphan Nuclear Receptors

The orphan nuclear receptors responsible for the regulation of drug metabolism genes include: the pregnane x receptor (PXR), the constitutive androstane receptor (CAR), the peroxisome proliferator activated receptors (PPAR), and the retinoid x receptors (RXR).

PXR, encoded by the gene NR1I2, is expressed predominantly in the liver and intestine and to a lesser extend in the kidneys and lungs. This tissue specific expression pattern resembles that of CYP3A and many compounds that lead to increased expression of members of the CYP3A family bind to PXR. This observation is what led to the understanding of the mechanism of PXR mediated expression of CYP3A4[81]. Many compounds, such as rifampin, dexamethasone

and omeprazole lead to increased expression of CYP3A4 through the binding of PXR[82]. PXR has also been shown to regulate the multi-drug resistance transporter MDR1 (ABCB1) [83].

CAR (NR1I3) was originally defined as constitutively active because it can form a heterodimer with RXR and bind retinoic acid response elements and activate target genes in the absence of ligands[84]. More recently, however, drugs such as HMG-CoA reductase inhibitors have been shown to activate CAR and lead to increased expression of CYP2B6[85].

There are three members of the PPAR nuclear receptor family: PPARα PPARβ and PPARγ. All three members have overlapping physiological function and are involved in the regulation of fatty acid metabolism through the induction of CYP4A[86]. Peroxisome proliferator drugs such as clofibrate, activate PPARα, which gives rise to increased expression of CYP4A, which in turn metabolizes the drug and reduces the exposure[76]. PPARγ can be activated by the thiazolidinedione class of drugs, rosiglitazone for example, that has been shown to increase the expression of the ABCG2 drug transporter giving rise to increase drug resistance [87].

Similar to PPAR, there are three members of the RXR family: RXRα, RXRβ and RXRγ. RXR is the common partner for all other orphan nuclear receptors in the formation of heterodimers. This heterodimer formation is a critical step for the binding and activation of all nuclear receptors[74]. There are two types of heterodimers, nonpermissive and permissive. RXR is silent in nonpermissive heterodimers and can only be activated by ligands of the partner nuclear receptor[74]. In permissive heterodimers, RXR can be activated by ligands of RXR or of the partner receptor[74]. Since RXR binds to most other nuclear receptors and affects the subsequent regulation of their targets, RXRs are involved in the regulation of most drug metabolism enzymes and transporters either directly or indirectly[77].

1.3.6.4 Nuclear factor-erythoroid 2 p45-related factor 2

Many phase II drug metabolism enzymes contain additional cis-acting regulatory elements such as the anti-oxidant response element (ARE) in their 5' regulatory region [88]. Nrf2 is the most potent activator of ARE-mediated transcription [88]. During chemical or oxidative stress, Nrf2 has been demonstrated to upregulate several phase II DMEs including UGTs and GSTs[88]. The exact mechanism of regulation of Nrf2 dependant ARE transcriptional regulation is unclear and likely involves multiple kinase pathways including the mitogen activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K) and extracellular signal regulated kinases (ERKs)[77]. Phosphorylation leads to the dissociation of Nrf2 from Keap1, a cytosolic inhibitor, followed by a subsequent translocation to the nucleus where it can exert its effects[88].

As part of my thesis, a thorough understanding of pharmacokinetic genes and pathways was undertaken in order to properly select and rank genes for inclusion in our genotyping assay. For the assay to be broadly applicable, genes from all of the various PK pathways described above were chosen, ranked and prioritized. These pathways are important for the metabolism of many different therapeutic classes of medications.

1.4 Pharmacodynamics

Pharmacodynamics (PD), or the effects of the drug on the body, is the study of the mechanism and pharmacological response to a drug. This involves the binding of drugs to receptors, post receptor effects and chemical reactions[4]. Drugs that bind to receptors exert their effects by either mimicking the binding of an endogenous ligand, leading to downstream activation, or by inhibiting the binding of a natural ligand and preventing the downstream

processes. Some drugs do not bind to a receptor but rather exert their effects through chemical processes, such as cytotoxic drugs.

No study of drug response or toxicity is complete without an investigation of the drug's PD profile, however in contrast to PK; PD pathways are, for the most part, unique to the drug in question. Thus, for a tool to be a broadly applicable across many therapeutic classes of medications, PK pathways are the most applicable.

1.4.1 KRAS and Epidermal Growth Factor Receptor Inhibitors

An example of variation in a PD pathway that affects drug response is the relationship between Kirsten rat sarcoma viral oncogene homolog (KRAS) variation and epidermal growth factor receptor (EGFR) inhibitor response. EGFR is a transmembrane tyrosine kinase that when activated by a ligand, leads to the activation of a series of signaling cascades to affect gene transcription[89]. This in turn results in increased cell proliferation, reduced apoptosis, metastasis and tumor induced angiogenesis[89]. Inhibitors of EGFR such as gefitinib or monoclonal antibodies that bind to EGFR such as cetuximab have been shown to be effective in the treatment of metastatic colorectal cancer and other cancer types[89].

One of the major downstream pathways activated by EGFR is the RAS-RAF-MAPK pathway. When variation in genes encoding members of this pathway were investigated for association with EGFR inhibitor response, it was found that mutations in KRAS were significantly associated with absence of response[90,91].This is because mutations that constitutively activate KRAS (located downstream in the EGFR-RAS-RAF-MAPK pathway) can bypass any and all effects of inhibiting EGFR[92]. Thus, knowledge of the variability in EGFR and KRAS (pharmacodynamic genes) can have a profound effect on the efficacy and choice of

drugs for therapy. Additionally, the overall survival of patients without a KRAS mutation was greater than those that were carriers[90,91].

While PD pharmacogenomic genetic variants are specific to specific medications and not generally applicable across therapeutic areas, they are still very important to measure for each clinical trial. In order to allow for the inclusion of project specific PD markers in the ADME genotyping panel, some genotyping space was purposefully left "open" in the panel design to be flexible. These flexible markers can be added/changed depending on the given needs of the project.

1.5 Genetics of Complex Traits

While selecting content to be included on the ADME panel, an understanding of current approaches in genetic research and clinical trials was needed to establish how current state-of-the-art methods and technologies are being used in order to maximally design strategies that would have the greatest impact and utility.

The ADME panel has been conceptualized to have two principle uses; as a tool for pharmaceutical clinical trials to direct decision making and to identity novel pharmacogenomic biomarkers that correlates with individuals with a specific drug response phenotype. Genetic studies of drug response are unique in that a specific exposure to the drug is required. This is why family based studies in pharmacogenomics are not common as it is unlikely to find large families where each member has received the same drug.

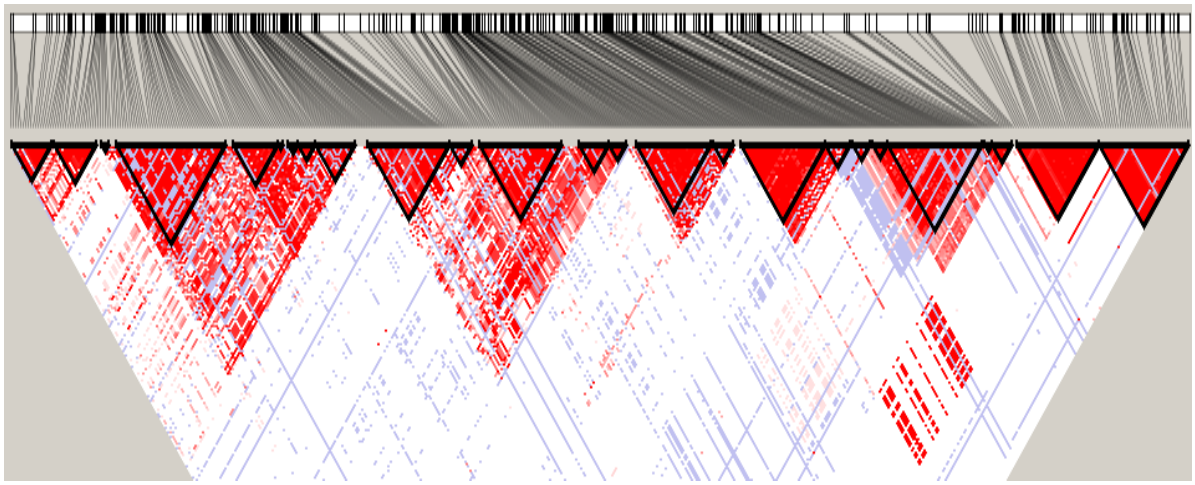
1.5.1 Candidate Gene versus Genome-wide

The two predominant strategies in genetic association studies are candidate gene and genome-wide genotyping. In the former, markers, usually single nucleotide polymorphisms

(SNPs) are targeted in regions in and around genes or genetic pathways that are candidates for association with a specific phenotype/disease being investigated. Typically, candidate gene studies involve the genotyping of a limited predefined number of markers (10's to 100's). The candidates are often selected based on hypotheses derived from previous studies or from knowledge of specific pathways. Conversely, a genome-wide approach involves the genotyping of hundreds of thousands to millions of well defined SNPs that span the entire genome that have been selected without any bias to specific regions or genes. Genome-wide association studies (GWAS) are non-hypothesis methods that hope to identify regions of linkage disequilibrium (LD) that associate with specific phenotypes being measured. No matter the study design, the causal variant may not be typed (direct association) but with a well designed study there is a good chance of identifying a region that contains the causal variant through LD with other markers present on the genome-wide panel (indirect association).

LD is defined as the nonrandom association of alleles at two or more loci[93]. LD between two loci will depend on the local recombination rate, as recombination that occurs between two loci on the same chromosome will break up the linkage between them. Alternatively, LD may arise for other reasons such as selection, where a particular allele may increases reproductive fitness, or non-random mating, where offspring receive two copies of the same chromosome. The combination of alleles on the same chromosome that are transmitted together is known as a haplotype. When multiple markers along a chromosomal region are considered simultaneously, often the LD extends to several markers. This set of loci in LD with each other is known as a haplotype block. It is possible to graphically represent stretches of LD along a particular region of the chromosome as haplotype blocks (see figure7)[94].

Figure 7: LD Map for a Region on Chromosome 1 Spanning the Gene DPYD in the CEU Population



Each black line represents the position of a single SNP (475 total). The pair-wise LD is calculated for each pair of SNPs and the value is translated into a colour. In the above example dark red indicates a D' value >0.9 and white indicated a D' value <=0.5. This region can be divided into 25 Haplotype blocks (black triangles). In order to capture the variation at all 475 SNPs using an r-squared of 0.8, 123 tag SNPs need to be genotyped. Figure generated with Haploview (*Barrett et al. 2005*).

In humans, haplotype blocks range from a few kilobases (kb) to more than 100 kb[95]. This discovery led to the hypothesis that testing for one marker or SNP, known as a tag SNP, within each LD block could be equivalent to testing every SNP in the block, thus significantly reducing the number of SNPs needed to interrogate the entire genome[96]. The opposite of LD is linkage equilibrium (LE), which implies that there is statistical independence between two or more loci. For LE to be reached, recombination needs to occur between two markers in LD. The shorter the distance between the two markers, the less likely that recombination will occur between them. This implies that it will take a very long time before high rates of recombination will ever be reached or even occur between two loci in strong LD and further supports the use of a tagging strategy[97].

In reality, the human genome is more complex than this simple LD block model, the recombination rate is not constant across the entire genome, some regions do not reflect the

block-like haplotype structure and the density of SNPs varies from region to region[98]. Further complicating matters is that different methods of defining LD lead to different block boundaries[99]. Despite this, the International Haplotype Project was carried out to characterize LD across the entire genome and catalogue common variation in 270 samples from four distinct ethnic populations; African (Yoruba in Ibadan, Nigeria; YRI), Asian (Han Chinese; CHB and Japanese Tokyo; JPT), and European (CEU) ancestry[100]. When phase two of the HapMap was released, the project contained data for more that 3.1 Million genome-wide SNPs [101]. More recently the project was expanded to include the genotyping data for an additional 1.6 million SNPs in 1184 individuals (the original four populations plus seven additional populations from around the world) that have resulted in the generation of a higher resolution map of variation across the genome[102]. Analysis revealed that haplotype block sizes vary significantly between populations. Shorter blocks where observed in African populations when compared to Asian and Caucasian populations. This is consistent with the out of Africa theory that states that there are many more unique chromosomes present in African populations and that when our human ancestors moved out of Africa more than 1 million years ago and spread around the world, only a limited number of chromosomes left the continent to define these other non-African based populations.[103,104].

While in the past, candidate gene studies and GWAS studies were performed independently, recently more and more genetic studies are starting to combine study designs into single experiments. There have been a number of successful examples where SNP tagging approaches, used predominantly in GWAS, have been successful applied to include specific candidate genes approaches. New analytical methods have been developed that allow for a focused analysis on candidate pathways even when whole genome genotyping is carried

out[105,106]. The statistical methods used for analysis are the same for both candidate gene and genome-wide association studies. Important aspects of theses analyses are detailed below.

1.5.2 Statistics in Genetic Association Studies

In order to ensure that the results of the statistical analysis are robust and not statistical artifacts, there are preliminary quality control measures and analysis considerations that need to be taken into account whenever statistical genetic analysis in undertaken.

1.5.2.1 Quality Measures

Data quality is very important in genetic association studies. The first area that can potentially lead to spurious associations is poor quality genotype calls. Many algorithms exist to translate the raw signal from genotyping technologies into base calls (see section 1.7). These algorithms are susceptible to anything that alters the signal output including DNA quality and concentration, or human manipulation errors. Genotyping data should be checked for unusual patterns such as batch effects (differences in allele frequencies between plates or among recruitment sites) or unusual patterns of missing data. Depending on the number of SNPs genotyped in the study, it may be unfeasible to visualize the clustering of homozygous and heterozygous calls of the calling algorithm which makes other quality checks even more important.

Another quality measure is to assess if each SNP is in Hardy-Weinberg equilibrium (HWE). HWE is true when the two alleles at a locus are not statistically associated. In other words, the allele frequencies in a population remain constant[107]. In theory, this is only true if there is random mating, no mutation, no exchange of alleles between populations, no selective pressure and an infinitely large population size. Since it is impossible in reality for a locus to be

in perfect HWE, generally, the practice is to remove loci that deviate from HWE at a significance level of 10^{-3} or 10^{-4} prior to carrying out the analysis. This may be too conservative however, because deviations from HWE can also be due to an association to the phenotype being investigated with a particular genotype[108].

1.5.2.2 Testing for Association

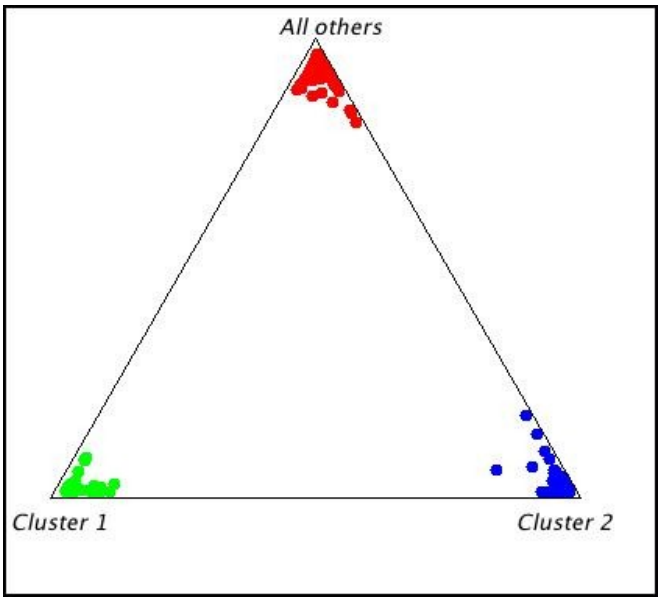
There are numerous statistical tests for testing for association between a single SNP and a phenotype, each with specific advantages and disadvantages such as increased power for a particular risk model or the requirement of certain assumptions. The statistical method used to for association will depend on the phenotype in question. In the simplest example, where the study involves cases and controls, one can test the null hypothesis that the counts of the three genotypes (heterozygotes and the two homozygotes) for a SNP do not differ between cases and controls. Tests such as the Fisher exact test, the Cochran-Armitage test and the Pearson test can be used in this case[107]. When a continuous quantitative phenotype in involved, a regression model can be used to test if there is a linear relationship between the mean value of the phenotype and the genotype. With regression models, it is also possible to take into account covariates such as age and sex that may be associated with the phenotype. In addition to single SNP analyses, multi-marker and haplotype approaches can also be carried out. These approached may be more helpful in determining the location of a causal variant[109].

1.5.2.3 Reasons for Spurious Associations

Ideally, a significant association between a SNP and a phenotype will arise because there is strong linkage between the SNP and the phenotype or the causal locus for the phenotype. There are however, two important causes of spurious associations that may not be accounted for by pre-analysis quality control of the data.

The most important cause is population structure. This will arise when a study involves multiple populations with a disproportional number of cases in one population. This will lead to any SNP with allele frequency differences between the outlying population and the others, becoming associated with the phenotype in question[110]. This was observed for example, with the association of a SNP in CYP3A4 and prostate cancer in African Americans that is no longer significant if population substructure is taken into account[111]. If the population structure of study participants is known, it can be taken into account by performing a stratified analysis[112]. Additionally, methods such as principal component analysis (PCA) can be used to assess population structure from genotype data that can then be used as covariates in the analysis (see Figure 8)[113,114].

Figure 8: Example of Population Stratification Structure Analysis Using HapMap Samples



Given a set of genetic markers that are not in LD, the program Structure[102] is able to detect the presence of distinct populations in samples and cluster them accordingly. In the above example, Cluster 1 are HapMap CHB and JPT samples, Cluster 2 are HapMap CEU samples and the "All others" cluster is HapMap YRI samples.

A second cause of false positive results is due to the large number of tests carried out. That is, when multiple SNPs are tested for association it is possible for the null hypothesis to be rejected by chance. The frequentist approach is to set a significance level α so that for any test, the probability of a false positive is α (usually 5%). If *n* SNPs are tested, assuming that the tests are independent, the appropriate per SNP significance threshold is approximately α/*n*. This is known as the Bonferroni correction[107]. While the Bonferroni correction is part of standard statistical design, this method can sometimes be too conservative if the SNPs tested are not independent, alternative methods such as permutation testing or the false discovery rate (FDR) approach can also be used to overcome the multiple testing problem[115].

All the methods outlined above were utilized during various stages of the development process and during the analysis of genetic data for this project.

1.5.3 Bioinformatics

As the size and scale of genetic association studies continues to grow, automated bioinformatic tools are necessary to complete most projects. They are required for assay and study design, to handle the vast amounts of data that is too large to manipulate by hand, and to perform the many types of complex statistical analysis that are required. Having a good understanding of what types of bioinformatics tools are available and how to automate data handling and analysis are becoming necessities in the present era of large scale genetic analysis.

For the ADME panel, two aspects of the assay design required the assistance of informatics tools. Firstly, a method was needed to select tagging SNPs for the hundreds of genes and in some cases entire loci included on the panel. There are several software packages designed for this process; however the algorithm we chose was LDSelect[96]. This software takes

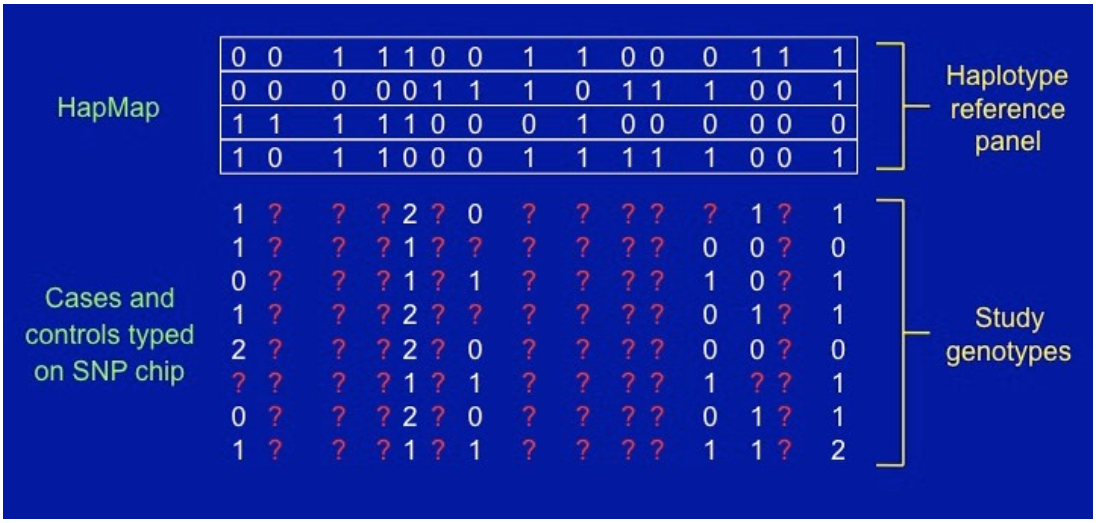
data from a reference population, in this case HapMap data, creates LD bins based on user defined criteria and selects one tag SNP per bin. An additional feature of the LDSelect algorithm is the ability to allow for input from multiple reference populations, which permits the selection of tag SNPs that will maximally cover LD bins in different populations[116]. For many LD bins, more than one tag SNP can be selected, when possible, other design features such as the homology and GC content of the surrounding sequence can be evaluated to help prioritize which tag to select.

For each iteration of the ADME genotyping panel, over 9000 primers needed to be designed. The primary program that is used by most researchers for designing primers is Primer3[117]. Although the tool is generic and intended to design PCR primers, various parameters can be manipulated to allow for the designing of different primer types under stringent conditions. Use of this tool was necessary but not sufficient for the complex designs that were required for the creation of the ADME panel (see section 1.7.1). Our complex primer design requirements led to the development of a tool, Optimus Primer (OP), to assist with our primer design needs. The OP tool enhances the capabilities of Primer3 and facilitates the design of large primer sets. This tool has broad applicability to next generation sequencing enrichment strategies and is being used presently by many groups (see section 5).

Approximately 295000 genotype calls are generated in a single run of the ADME panel (96 samples). As more and more runs are performed for any given study, it becomes clear that the amount of data generated becomes too large to handle with standard spreadsheet and text manipulation software. There are many data formats that have been and are being developed for the handling of genomic data, many of these tools, such as CASTOR-QC and MedSavant utilize the features of modern database software to store and manipulate data[118,119].

Once the data is generated and stored, it needs to be analyzed. Depending on the study, many different quality and statistical approaches may be performed (see above). Freely available statistical packages such as R are very good for basic statistical and graphing functionality; however their performance declines as the data sets become increasingly large [120]. One utility that is commonly used in genetic studies of any type is PLINK[112]. PLINK is an entire tool set of utilities that can be used in genetic studies. Common features include: data filtering based on minor allele frequency (MAF) or HWE, association statistics including regression models, and calculations of genome-wide identity-by-state to detect relatedness in your sample set.

Figure 9: Overview of Imputation of Genotype Data



Source: *Marchini et al. 2007*

Genotypes for a particular locus are depicted as a 0 or 1 for the two possible alleles. The linkage disequilibrium pattern from a reference panel, such as the HapMap, is extrapolated to the study samples to fill in genotypes of untyped markers (depicted as ?). Additional information such as the recombination rate along the genome can also be incorporated to facilitate the imputation[114].

One of the most recent advances in the analysis of genetic data is the incorporation the method of imputation. Imputation is a method to determine allele calls for SNPs that are not genotyped in a given study population. Imputation expands a studies data by increasing the

number of SNPs for analysis by including SNPs that were not directly genotyped but whose genotype can be imputed by accessing LD patterns from large reference population data sets. This method of analysis has been shown to add power to genetic studies by boosting the association signal obtained[121]. Some software packages that are used for imputation include BEAGLE and IMPUTE2 which will generate probability vectors for the three possible genotypes[122,123] (see Figure 9). Certain statistical programs such as SNPTEST, allow for the uncertainty in probability distribution of the imputation results to be incorporated into a test for association[122].

In the work presented within this thesis, imputation was used as a quality measure to strengthen association results observed. Association results with a greater number of signals after imputing additional SNPs were considered to be more reliable.

1.6 Pharmacogenetics and Pharmacogenomics

Pharmacogenetics has been defined as the study of individual gene variation on drug response while pharmacogenomics involves the investigation of the entire genome including downstream processes such as transcription. Often the terms are used interchangeably. Pharmacogenomics or pharmacogenetics, in the broadest sense of the terms, refers to the study of the relationship between heritable variation and inter-individual variation in drug response[124]. The concepts of pharmacogenetics were first introduce in 1957 by Arno Motulsky who suggested that drug sensitivity, to drugs such as the anti-malarial drug primaquine and the muscle relaxant succinylcholine, may be produced by similar genetic mechanisms, however the term was not coined until 1959 by Friedrich Vogel[125,126]. Pharmacogenetics became a discipline on its own following Werner Kalow's 1962 monograph which outlined the field[127].

Early pharmacogenetic research focused of variants with Mendelian-like effects on response. One of the more famous examples arose in 1977 when five members of a laboratory at St Mary's Hospital Medical School in London took the drug Debrisoquine. One of the participants was Robert Smith, who was the head of the lab, and within hours began to feel dizzy and disorientated, and eventually collapsed with sever hypotension. While in most people debrisoquine is rapidly metabolized to an inactive metabolite and eliminated in the urine, Smith's urine contained no metabolites of debrisoquine[128]. The work eventually led to the cloning of the gene responsible for the metabolism of debrisoquine, cytochrome P450 subfamily IID polypeptide 6 (CYP2D6)[129].

The genes that encode for most drug metabolizing enzymes contain variants that have been demonstrated to influence enzymatic activity. Many of these functional variants have been associated to variation in drug response. When Phillips and colleagues looked at 27 different drugs prone to adverse drug reactions, they found that 59% of the drugs were metabolized by at least one drug metabolizing enzyme with known functional variants, whereas only 22% of randomly selected drugs satisfy this criteria[130].

For many ADME genes frequently studied in pharmacogenomic studies, the star (*) nomenclature has been established as naming scheme for genetic variants. For example this is the standard used for cytochrome P450s[131]. The difficulty with this type of naming scheme is that this nomenclature often defines haplotypes, and while many of these haplotypes are defined by a single marker, in some cases they are not. Although this information is relevant, its usefulness is derived by its presence in a defined haplotype and often requires translation tables to define a specific star phenotype from a variable set of genotypes.

1.6.1 Pharmacogenomic Examples

Many pharmacogenomic examples have the necessary evidence to have real clinical relevance and regulatory agencies such as the US Food and Drug Administration (FDA) and Health Canada have made recommendations for genetic testing in patients being treated with these drugs (see appendix 1)[132]. With of all these mature examples (some of which are detailed below), knowledge of the pharmacokinetics and pharmacodynamics of the drug led to the eventual discovery of genetic variation that gives rise to variation in response. These examples demonstrate the utility of investigating genetic variation in know relevant pathways. Additionally, the following examples show the type of evidence needed in order to consider a genetic variant for clinical guidance and decision making.

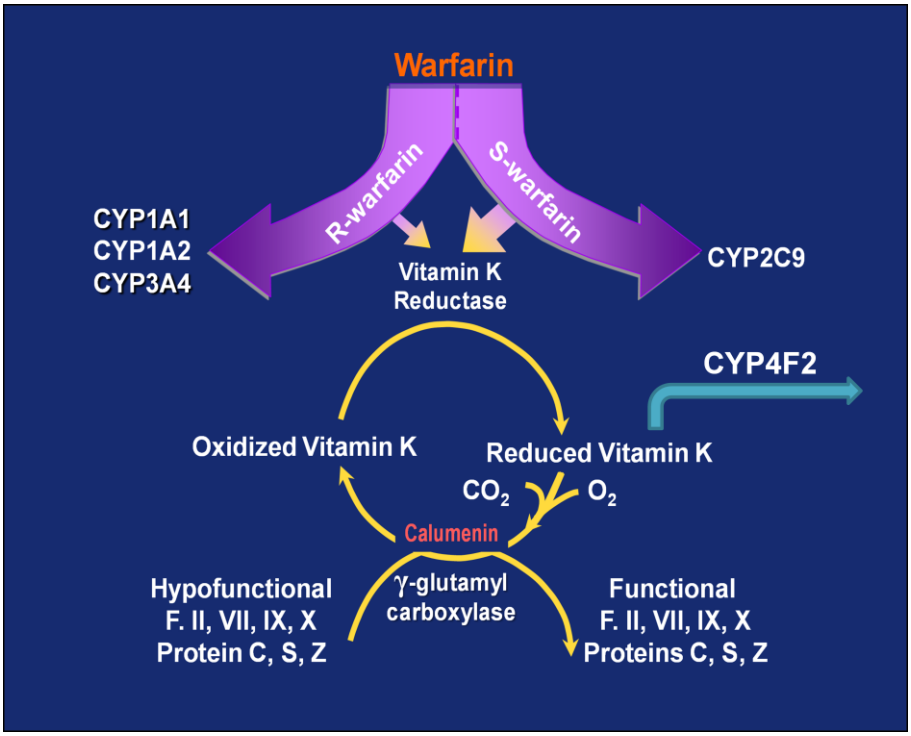
1.6.1.1 Warfarin

Perhaps one of the best known pharmacogenomic examples is the genetics of warfarin dosing. Warfarin has been on the market since 1954 and despite its extensive use, the mechanism of action was not elucidated until 1978. In 1974, *Stenflo et al.* showed that Vitamin K was a cofactor for the post translational carboxylation of specific clotting factors. Vitamin K is then transformed into Vitamin K epoxide[133]. The epoxide derivative is then recycled back to the reduced form of vitamin K by vitamin K epoxide reductase (VKOR) in what is known as the Vitamin K cycle[134] (see Figure 10). *Whitlon et al.* showed that Warfarin exerts its effect by inhibiting the VKOR enzyme thus reducing the available Vitamin K to serve as a cofactor, and blocking the activation of Vitamin K dependant Clotting factors[135]. The gene encoding for VKOR was not discovered however until 2004 by *Li et al.*[136] The effects of Warfarin can take up

to several days to elucidate as the stores of previously activated clotting factors become diminished[137].

There are several clinical indications for when a patient should be prescribed warfarin, they include: atrial fibrillation, artificial heart valves, and deep vein thrombosis. Many additional clinic indications that are at risk of thrombolytic events use warfarin to reduce the capability to form a blot clot, to reduce the risk of embolism, or the spread of a thrombus. While the benefits of the prescribed treatment are effective, warfarin remains one of the most dangerous drugs on the market because it is difficult to dose correctly[138]. This is due to the huge amount of variability among individuals with regard to the response to the drug[137]. Currently, when

Figure 10: Warfarin PK and PD



Adapted from *Gage et al. 2005*
Warfarin exerts it effect by blocking the conversion of oxidized vitamin K to the reduced form through vitamin K reductase. The reduced vitamin K is needed as a co-factor in the activation of many clotting factors.

patients are first prescribed warfarin, treatment is individualized by close monitoring of the patients International Normalized Ratio (INR). The ratio measures the time it takes blood or plasma to form a clot after the addition of a tissue factor, otherwise known as prothrombin time (PT). Because the reaction can vary depending on the manufacturer of the tissue factor, the test has been standardized by an International Sensitivity Index (ISI) that the manufacturer assigns to a given tissue factor. The INR is therefore the PT of the patient divided by the PT of a "normal" control, raised to the power of the ISI. This ensures that the INR of a patient is the same no matter what lab carries out the procedure and what tissue factor is used[139]. The desired INR of a patient receiving warfarin therapy is generally between 2.0 and 3.0, which is a very small therapeutic range[140]. Warfarin is considered a drug with a narrow therapeutic index in that the minimum toxic concentration is less than twice the minimum effective dose. In essence, physicians play a game of trial and error in attempting to raise the patients INR to the desired range. Despite the additional monitoring of warfarin treatment, the incidence of adverse drug reactions is estimated to be between 7.6 and 16.5 per 100 patient years which is very high for such a commonly prescribed medication[134].

Warfarin is comprised of two enantiomers, an R and an S form. The S form is 5 times more potent than the R as an inhibitor of the vitamin K reductase[141]. The S form is primarily metabolized by CYP2C9 and several functional variants have been identified, the two most important being the CYP2C9*2 and CYP2C9*3 mutations[142–145]. Several studies have shown that individuals homozygous for these mutations can have as little as 5% of normal enzymatic activity and require a lower mean daily dose and are more prone to adverse bleeding events[146]. The frequency of the *2 and *3 alleles in non-Caucasian populations is low and other functional

variants are likely to contribute to warfarin dosing variability, these include *5, *6, *8 and *11[147].

As mentioned above, warfarin acts by inhibiting the enzyme responsible for the recycling of Vitamin K in the Vitamin K cycle. The enzyme VKOR is comprised of several subunits and it has been shown that sub unit 1 (VKORC1) is primarily responsible for the VKOR activity. Missense mutations in this gene seem to explain what is known as warfarin resistance, perhaps due to the fact that warfarin is unable to bind to the subunit and exert its effects[148–150]. However, like CYP2C9, several common SNPs in VKORC1 have been associated with warfarin sensitivity. Specifically, the markers that define two haplotypes (labeled A and B or H1 and H2 or *2 and *3 depending on the study) have been associated with low and high dose requirement of warfarin[151–155]. It has also been shown that these haplotypes correlate with variable VKORC1 mRNA expression levels which in turn translates to variability in VKORC1 receptor numbers that now sheds light on part of the mechanism responsible for warfarin sensitivity[153]. Taken together, in the above mentioned studies, about 40% of warfarin dose variability can be explained by variation in CYP2C9 and VKORC1. When other clinical factors such as age, weight, height and interacting drugs are also considered, over 50% of the variability in dose can be explained in Caucasian populations. These results are similar in Asian populations, however they do not explain as much of the variation in African populations[156,157].

More recently, the role of CYP4F2 in the vitamin K cycle was established as the primary liver microsomal vitamin K oxidase that removes vitamin K from the cycle[158]. A single variant in CYP4F2 (V433M) has been associated with warfarin dose variation in multiple studies[159–162]. CYP4F2 accounts for an additional 1-4% of warfarin dose variability, however the clinical utility of genotyping CYP4F2 is presently unclear.

The final member of the vitamin K cycle to be investigated for association with warfarin dose is gamma glutamyl carboxylase (GGCX). One SNP (rs11676382) has been associated with warfarin dose variability in Caucasians and African-Americans but it only contributes to a lesser extent and is not considered for most dosing algorithms[163]. Other SNPs identified in GGCX have conflicting data as to their effects on warfarin dose.

Many studies are now taking both genetic and clinical factors into account and have created warfarin dosing algorithms that can help guide dosing[164]. Theses algorithms are constantly being updated to include new findings. Several dosing algorithm are presently being evaluated to prove their utility in the clinical setting[165]. *Epstein et al.* genotyped 896 cases matched to 2688 historical controls and over a 6 month follow up period, prospectively found that genetic testing reduced hospitalizations by 31% overall and bleeding and thromboembolism hospitalizations were reduced by 28%[166]. Thus, the potential for pharmacogenomics to improve healthcare is now becoming a reality.

1.6.1.2 Clopidogrel

Clopidogrel is one of the most widely prescribe anti-platelet medications that acts to inhibit the formation of blood clots by inhibiting platelet aggregation and goes by the trade name Plavix[167]. Clopidogrel is a prodrug and must be metabolized to produce the active metabolite. This active metabolite selectively inhibits the binding of ADP to its platelet receptor (P2Y12) and prevents the ADP mediated activation of the glycoprotein complex that is the major receptor for fibrinogen[168]. Thus, the mechanism of action for clopidogrel ultimately inhibits the P2Y12 platelet receptor so that fibrinogen cannot bind to platelets to cause aggregation.

Several genes have been investigated for association with response to clopidogrel. These include: the gene coding for efflux transporter ABCB1, involved in the intestinal absorption of clopidogrel; the P2Y12 receptor gene; and several CYP genes involved in the metabolism of clopidogrel[169,170]. The only gene that consistently shows association with primary clinical outcomes is CYP2C19[171–175]. CYP2C19 is involved in both the formation of the active metabolite and the 2-oxo intermediate metabolite[167]. A number of polymorphisms in CYP2C19 have been associated with clopidogrel response; of note are the alleles that define the *2 and *3 loss of function haplotypes whose carriers have reduced clopidigrel response[170,171]. Another allele is *17 which early data indicates results in increased enzyme activity and enhanced metabolism of clopidigrel and therefore increased platelet inhibitor and increased risk of bleeding[176]. This result has not been replicated as yet. Several cohort and post-hoc clinical trial studies have evaluated the effects of CYP2C19 variation on both the clopidigrel response and adverse events. The diminished anti-platelet response to clopidigrel for patients that are CYP2C19 intermediate and poor metabolizers has been observed in over 20 studies consisting of over 4500 subjects[177]. Adverse cardiovascular events have also been associated with the CYP2C19 genotype. In two studies, patients with impaired metabolized status had up to a 2.7 fold increased risk of new cardiovascular events (death, myocardial infarction, stroke or urgent revascularization)[171,173]. Patients with impaired metabolizer status have also been observed to have a 2.1 to 2.8 fold increases risk of stent thrombosis[172–174].

Interestingly, two more recent studies have led to conflicting results. The first, involving two large randomized placebo-controlled trials of clopidigrel for treatment of acute coronary syndrome (ACS) and atrial fibrillation, showed that impaired metabolizer status for CYP2C19 had no effect on risk of cardiovascular outcomes[178]. This may be attributed to the fact that only

14.5% of patients in these trials underwent percutaneous coronary intervention with stenting compared to 70% in previous studies. The second, a genetic substudy of a clinical trial comparing ticagrelor versus clopidigrel in the treatment of ACS, found that patients with impaired metabolizer status for CYP2C19 had increased risk for major cardiovascular events but only in the first 30 days of treatment[179]. Taken together these results suggest that genotyping for clopidigrel therapy may be only important in acute situations where patients are undergoing stenting. Despite these results, two recent meta-analyses, looking at almost 20 000 patients, show that there is a strong association between CYP2C19 loss of function alleles and adverse cardiovascular events with even greater risk when stent thrombosis is analyzed separately[180,181].

1.6.1.3 Thiopurine Drugs

Thiopurine drugs, azathioprine (AZA), 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG), are purine antimetabolites used in the treatment for acute lymphoblastic leukemia and other hematological malignancies, autoimmune disorders such as Crohn's disease and Rheumatoid arthritis, and for the prevention of organ transplant rejection. All three drugs are given as prodrugs and require intracellular activation via multiple metabolic pathways to give rise to their cytotoxic effects[182,183]. The active metabolites can exert their effects in a number of ways. They can be incorporated into DNA or RNA, leading to the inhibition of replication and DNA and RNA repair processes, they can prevent the *de novo* synthesis of purines and finally they can inhibit Rac1 which leads to apoptosis in T-cells[184]. The pathways that lead to the production of active metabolites are in competition with the pathways that lead to the inactivation and clearance of the drugs. Specifically, the inactivation is catalyzed by two enzymes, xanthine oxidase (XO) and thiopurine methyltransferase (TPMT)[182,185].

The catabolism reaction by XO occurs primarily in the plasma and urine following thiopurine administration. Xanthinuria is a rare genetic disorder that is caused by a deficiency in XO and leads to renal failure. Aside from this rare disorder, there is a 4 to 10 fold difference in inter-individual XO activity in individuals[186,187]. To date, three small studies have looked at genetic variation in XO in relation to thiopurine response or adverse reactions and the results have been inconclusive[188–190]. One study found a weak protective effect of the C837T SNP (p =0.048) while another showed a trend to higher 6-MP concentrations in patients heterozygous for two other variants[189,190]. Further studies are required to investigate genetic variation in XO.

The enzyme that is most studied with established evidence of the role genetic variation as a contributor to inter-individual variability in thiopurine efficacy and toxicity is TPMT. Deficiency of TPMT was first described in 1980 when it was observed that 1 in 10 Caucasians have intermediate enzyme activity and 1 in 300 have deficient enzyme activity[191]. Over the next 20 years, several studies documented the association between TPMT activity and the levels of cytotoxic metabolites and the relationship between TPMT activity and severe myelotoxicity[192–195]. In general, TPMT deficiency is associated with more severe myelotoxicity, although only 25% of all cases of thiopurine-induced myelosuppression can be explained by TPMT deficiency, and increased TPMT activity is associated with poor treatment response and higher risk of relapse[196,197]. Recently, two meta-analyses have been carried out to summarize the relationship between TPMT variation and thiopurine response and toxicity. These analyses showed a 2.93 fold increase (95% C.I. 1.68-5.09) in the incidence of patients who were homo or heterozygous for the TPMT *3A and *3C variants and who had thiopurine-induce adverse reactions[198]. Additionally, the odds ratio for leucopenia in patients that with reduced TPMT activity was 4.19

(95% C.I. 3.20-5.48) indicating the potential value of TPMT genotyping to reduce adverse drug reactions[199].

1.6.2 Genome wide Association Studies in Pharmacogenomics

There have been many genome wide association studies (GWAS) performed on drug toxicity and response phenotypes since 2005, when the first GWAS studies were published[200]. The majority of these studies have not found any statistically significant associations[201]. There are a number of reasons why pharmacogenomic GWAS studies have not been very successful. First, it is difficult to recruit the large sample sizes needed to have statistical power to detect small or medium sized effects. In contrast to complex-disease phenotypes, an exposure to a particular drug is necessary to give rise to a drug response phenotype, and patients that are non-responders or have an adverse drug reaction are rare compared to patients that respond favorably to the drug. This also makes it difficult to replicate any findings that may be observed in a second cohort of patients. Second, the characterization of drug response phenotypes can be very difficult unless there is a clear quantitative measure as is the case with some drugs such as anti-coagulants. Furthermore, many of the GWAS studies that have been conducted have been performed on clinical patient samples years after the trials have been completed and much of the phenotypic data is often incomplete or was not captured due to the fact that the trials were not designed as genetic studies. This gives rise to heterogeneous phenotypes that mask true associations. Lastly, the coverage of current genome-wide SNP arrays is not complete and specifically rather poor in ADME gene regions. Due to reasons such as SNP density and homology present in many pharmacologically relevant genes, genetic variation in most known ADME candidate genes is frequently not fully tested (see below).

Despite the added difficulties of performing a GWAS study on pharmacogenomic phenotypes, there have been a number of successful examples reported to date (see table 5).

Table 5: Published Successful Pharmacogenomic GWA Studies.

Drug	Phenotype	Number of Cases	Associated Gene(s)	Reference(s)
Warfarin	Maintenance dose	181; 1053	VKORC1, CYP2C9,CYP4F2	202,203
Clopidogrel	Antiplatelet effect	429	CYP2C19	204
Interferon-α	Response in hepatitis C infection	1137; 293; 154	IL28B	205–207
Methotrexate	Drug clearance	434	SLCO1B1	208
Simvastain	Myopathy	85	SLCO1B1	209
Flucloxacillin	Liver injury	51	HLA-B*5701	210

There are two interesting points to note when looking at genes associated with drug response and toxicity phenotypes in GWA studies: firstly, in four of the six studies, the only associated genes are ADME genes and secondly, in the warfarin and clopidagrel studies, the genome-wide approach did not add any additional information to what was already know using a hypothesis driven candidate gene approaches.

1.6.3 In Vivo Probe Drugs

While many genetic variants have been associated with drug response, there remains variability in drug response that cannot be explained by genetics alone. Additionally, genetic variability does not account for other factors that influence drug response such as environment or disease status[211]. The only method to accurately assess the in vivo activity of drug metabolism

enzymes and transporters is with the use probe drugs. Probe drugs are known substrates for a particular enzyme that are administered and following the appropriate measurement of pharmacokinetic parameters, are used to determine quantitatively enzyme or transporter activity[212]. The most common form of in vivo probe phenotyping, involves the administration of a drug followed by the measurement of the drug's metabolite in plasma or urine. The usual method of choice for metabolite quantification is chromatographic separation with tandem mass spectrometry[212]. A number of compounds have been validated as probes for several phase 1 metabolism enzymes. These include: caffeine for CYP1A2, warfarin for CYP2C9, omeprazole for CYP2C19 dextromethorphan for CYP2D6 and midazolam for CYP3A isozymes. Probe drugs for phase 2 metabolism enzymes and transporters are not as well developed and not as frequently used however include caffeine for NAT2 and digoxin for ABCB1 [213,214]. While the administration of a single probe drug may be useful for selective phenotyping of a single enzyme, efforts have been made to create combination probes, or cocktails, of probe drugs to simultaneously assess the activity of several metabolism enzymes. Examples of probe cocktails include: the Cooperstown 5+1, for cytochrome P450 1A2, 2C9, 2C19, 2D6, and 3A, *N*-acetyltransferase-2, and xanthine oxidase; and the Inje cocktail that uses probe drugs that are more readily available for the assessment of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A[215,216]. There are limitations to the cocktail approach however; including the potential for interaction between probe drugs, side effects and toxicities of probe drugs and analytical complexities is assessing several metabolites concurrently which is why the maximum number of enzymes that can be quantified simultaneously using this method remains low[217]. This is a why a genotyping approach remain the only method for broad characterization of several ADME pathways at once.

1.7 Genotyping Technologies

When selecting a genotyping technology for the ADME panel, it was important to evaluate all the technology platforms for a number of critical parameters; throughput in terms of number of SNPs and number of samples that can be processed simultaneously, accuracy, consistency of the data, ease of use, and cost. Thus, we were looking to identify the optimal technology and platform that is highly accurate but which balances throughput with costs. There are several available technologies on the market today, each with their own advantages and disadvantages depending on the application. However, no matter the technology, there are two features common to all platforms; they must have a method/technology that can differentiate between a minimum of two alleles and that the different alleles produce unique signal profiles that can be interpreted by genotyping calling algorithms[218].

The most common genotyping methods commonly used to discriminate alleles are single primer extension, hybridization, and ligation. In single primer extension reactions, an enzymatic reaction involves the allele specific incorporation of dideoxy-nucleotides to extend a primer bound to a DNA template by one base. Generally, a unique primer is designed to bind with its 3' end adjacent to the SNP being interrogated, thus the allele discrimination is determined by the enzymatic addition of a single labeled nucleotide. The label is incorporated into the extension product, the product is isolated and some sort of detection is performed. The label can be a mass and detection can occur by means of mass spectrometry (MassExtend iPlex arrays; Sequenom; San Diego, CA), or the label can be a flurophore that when excited, emits light of a certain wavelength that can be detected (SNPstream; Beckman Coulter, Fullerton, CA & Infinium assay;

Illumina, San Diego, CA). In hybridization based reactions, the reaction conditions are such that probes will only bind to perfectly complimentary targets. Thus, specificity is determined by the binding of probes to the allele in question. In this type of reaction, a probe is required for each possible allele of the SNP in question. Generally, the different probes will have different labels similar to the primer extension reactions or the probes may be anchored to a solid state such as a microarray (Affymetrix Genechip microarrays, Santa Clara, CA). In ligation reactions, the specificity is determined by both hybridization to unique DNA sequences and the specificity of the enzymatic activity of the ligase enzyme that can only ligate primers that are located proximally to each other. Typically, three probes are needed for this type of reaction; two probes are allele specific that are complimentary to the variant nucleotide and the third binds in the adjacent region to the SNP that is being queried (GoldenGate assay, Illumina, San Diego, CA). The ligase will then only join the probes that are perfectly complimentary creating a single oliognucelotide. The detection occurs in a similar fashion to the primer extension method with differently labeled allele specific probes.

The majority of assays on the market use one or a combination of the detection methods described above. The level of plexing (number of SNPs interrogated simultaneously) tends to be inversely proportional to the number of samples that can be run simultaneously in a single experiment. At one extreme are the whole-genome genotyping arrays, such as the Illumina Infinium Beadchips which use a primer extension reaction of probes anchored to beads and the Affymetrix GeneChip arrays that use a hybridization approach. Currently, these assays can interrogate millions of SNPs in a single experiment. On the lower end of the plexing spectrum are technologies that do not interrogate as many SNPs simultaneously; however these technologies are able to process hundreds of samples in a single experiment. One example is the

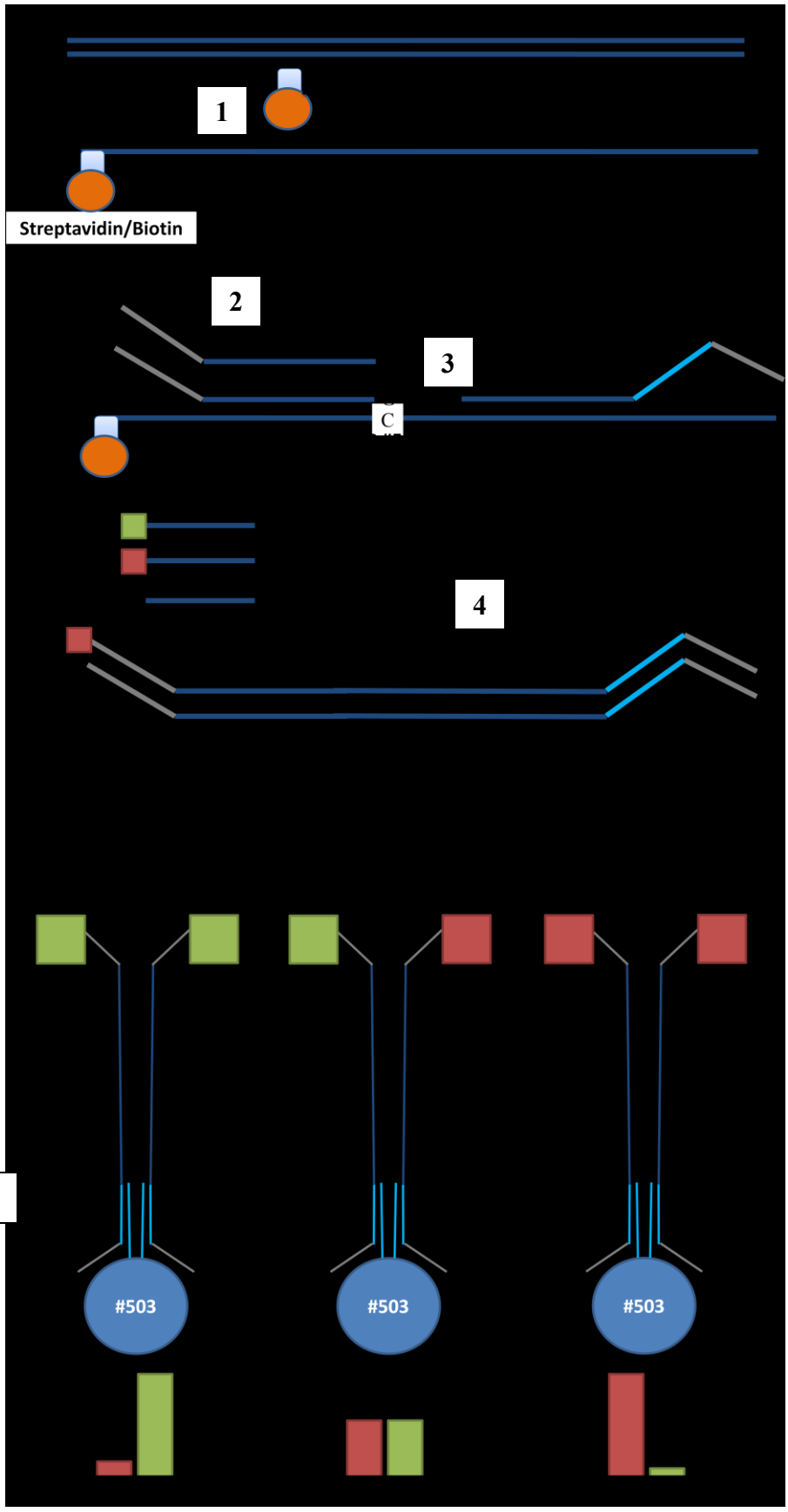
Sequenome iPlex assay that query up to 40 SNPs simultaneously in a single reaction, using a single primer extension reaction, in up to 384 samples in a single experiment.

1.7.1 The Golden Gate Platform

The platform selected for the ADME panel development was the Illumina GoldenGate (GG) assay[219]. The GG assay analyzed on the Bead Station platform allows for the plexing of up to 3072 SNPs in a single reaction and can process 96 samples in a single experiment. An advantage of the GG platform is that the allele discrimination chemistry is highly accurate and consistent because it incorporates three levels of specificity; hybridization, primer extension and ligation. The reaction proceeds as follows (see Figure 11). 1. Genomic DNA is activated through a biotinylation reaction and is immobilized on streptavidin beads. 2. A pool of oligonucleotides (OPA), containing three primers per SNP, is added to the activated DNA. The three primers are designed as two allele specific primers (ASOs) that will only anneal to DNA that is 100 percent complimentary and a locus specific primer (LSO) that will anneal a few bases 3' to the SNP in question. 3. A primer extension reaction is performed next that will extend the ASO that has successfully bound towards the LSO and a ligation reaction further joins the extended ASO to the LSO. Unextended primers, excess reagents and the genomic DNA are washed away leaving only a pool of ligated ASO-LSO oligos. 4. The ASO and LSO contain sequences that allow for universal PCR amplification using universal primers that incorporate one of two possible fluorescence labels into the amplified product. The LSO also contains a sequence (illumacode) which is complimentary to a probe anchored to a bead on a Sentrix array or on a GoldenGate chip. 5. The PCR product is then hybridized to the bead and the fluorescence is detected to determine genotype.

Having multiple levels of specificity, along with the number of SNPs that can be included and run on 96 samples at a time made the GoldenGate platform ideally suited for the ADME panel development.

Figure 11: The GoldenGate Chemistry



The goal of this introduction was to provide an overview of the background that was researched in to order design, optimize and complete the development of a broad base ADME genotyping panel that has broad applicability to many research and clinical studies. Furthermore, the introduction was also intended outlined the extensive bioinformatic and statistical genetic tools that were necessary to adequately interpret and analyze our large data sets. Lastly, the introduction gave an overview of the current state of pharmacogenomic research and demonstrated that there is an unmet need for the creation of a targeted broad based ADME panel the can be used in clinical research that can simultaneously screen known functional variants but at the same time identify novel pharmacogenomic biomarkers in key ADME genes.

The following sections of this thesis are organized into 4 additional chapters. Chapter 2 describes how the genetic content, both on the gene and SNP level, for the ADME panel was selected in collaboration with the PharmaADME working group; made up of members of the pharmaceutical industry and academia. Chapter 3 details the design, development, optimization and validation processes that were perform to complete the development of the 3000 SNP broad based ADME genotyping assay. Chapter 4 details the utilization of the assay in a research project involving DNA samples derived from human liver tissue in order to demonstrate the utility of the assay. Finally, Chapter 5 details how a bioinformatic tool that was developed in support of the ADME panel assay development process was further developed and made publicly available.

2 Development of a Consensus List of ADME Genes and Polymorphisms for Use in Pharmaceutical Clinical Trials: The ADME "Core" List

This section describes the process of selecting the content for the ADME panel. In order to facilitate this process, in addition to extensive literature searches, the expert opinions of 7 pharmaceutical and 3 life-science companies was sought to develop a consensus list of ADME genes for which there is compelling evidence of relationship between genotype and ADME pathways, and which could be used as a standard panel in drug development and to support regulatory submissions, and, if appropriate, general clinical practice. Through an iterative process, we identified a "Core List" of 32 ADME genes meeting these criteria, and 184 markers comprising all currently known alleles with utility in testing clinical samples.

This process was unique because in addition to tapping into published resources to select ADME genes and variation, detailed information from the drug development programs of several pharmaceutical companies was incorporated. Not all of this information is available to the public and therefore it provided this initiative with a thorough list of ADME genes and variation, one of the most complete to date.

The content described in this section formed the basis for the ADME panel development, described in section 3. Interestingly, three genomic technology companies have used the information generated in this collaboration to develop ADME genotyping assays. Being the curator of the data allowed me to analyze and merge the data from the various sources into a single universal format. Additionally, when possible, the information had to be verified either through resources such as the Human Cytochrome P450 (CYP) Allele Nomenclature Committee, dbSNP and often the original publication describing the gene or variation[220,221].

Finally, a website was created to store and share everything generated in this initiative. Access to this website is freely-available to the entire pharmacogenomic community that can be accessed using the following URL: www.pharmaadme.org. Importantly, the website also serves as a portal for discussion so that we can keep the data up to date.

2.1 Introduction:

The advent of high throughput genetic analysis technologies and the ability to identify genes which can modify risk for disease, affect response to therapy or predict the development of side effects have been espoused as a means to improve drug development and ultimately therapeutic outcomes[222]. Many terms, such as personalized medicine, tailored therapy, or "the right drug into the right patient", are often used to describe how applied genetics will benefit and improve health outcomes in patients. However, as the last several years have demonstrated, the maturation of genomic associations into successful genomic biomarkers and then dissemination of these observations into general medical practice requires multiple, time consuming steps. Presently, one of the major goals of the clinical research community is to find ways to effectively translate this information into improved patient care.

2.2 FDA Critical Path:

In order to address documented inefficiencies associated with drug development, the FDA, in March 2004, outlined their proposal for the critical path initiative to accelerate the drug development process[223]. Among other aspects, the proposal identified some of the major challenges associated with the current drug development pipeline and outlined *the need for better evaluation tools*. To this end, the FDA recommended the development and implementation of

programs to accelerate the identification of new biomarkers that can improve clinical outcomes by helping to predict safety and drug response profiles[223]. With the recent advances both in our knowledge of the human genome and in genomic technologies, researchers are now starting to understand the role that genetic variation plays in a drug's metabolism and/or mechanism of action[224]. One goal is to better understand how variation in genes involved in metabolism and transport affects how patients respond to specific drugs. Improved predictive genomic tools may be used to select which patients receive a particular drug, how much of that drug is prescribed, and at what frequency and duration. It is anticipated that the use of genomic biomarkers will have a profound effect on the way drugs are developed and subsequently utilized, by producing tools that identify genomic factors that can affect a drug's efficacy and safety.

2.3 The Pharmaceutical Company Perspective

Many pharmaceutical companies have begun to integrate biomarker development programs into their pre-clinical and clinical drug development pipelines with the goal of improving drug development and impacting patient outcomes[224,225]. Recently, genomic biomarker programs have seen success in measuring DNA variation and/or changes in gene expression and relating that to adverse drug response or disease progression and risk[226–228]. These genomic approaches are becoming more widely incorporated into drug development, and have been facilitated by better technologies, the completion of the sequencing of the Human Genome and by the HapMap project[229–232].

Genetic biomarkers predicting improved therapeutic benefit can fall into one of three categories: 1) predicting likely response to therapy; 2) predicting susceptibility to an adverse event; or 3) predicting an altered metabolic profile. Furthermore, the marker must be

sufficiently validated and the risk conferred by the marker must be sufficiently understood. In addition, a validated assay, with defined sensitivity and specificity, must be generally available to meet the standards necessary for modern medicine. Finally, if a validated biomarker is to be co-developed with a drug or to be used to direct prescribing practices, regulatory approval is mandatory.

Variability in individual patient response to a drug, in terms of therapeutic benefit and/or side effect profiles, is commonly observed for many marketed pharmaceuticals. Genetic variation at the drug's molecular target or within critical components of the target molecule's pathway might be the cause of some variable responses to administered drugs. Alternatively, variation in genes encoding key enzymes involved in pathways affecting pharmacokinetics (PK) may directly contribute to the observed variation in patient response to drug. While we acknowledge the potential importance of the former source of variable drug response, we are particularly interested in the latter, i.e. the pathways that affect how a drug is Absorbed, Distributed, Metabolized and Excreted (ADME). One area of genetic biomarker research where there has been steady flow of published information is the study of the effects of genetic variation on these pharmacokinetic pathways[228,233,234]. This is of particular interest because, for the most part, drugs are all metabolized by the product of a well-known set of genes and pathways that influence their pharmacokinetic responses while the mechanisms of action and the therapeutic uses of the various drugs may differ.

For example, *Phillips et al.* observed that 59% of the 27 drugs most frequently cited in Adverse Drug Reaction (ADR) studies were known to be metabolized by an ADME enzyme with at least one known loss of function allele[235]. With the advancement of today's technology platforms, expertise to develop genomic tools that can help identify variants in ADME-related

genes affecting pharmacokinetics, and thus efficacy, safety, and dosing of a drug has become a cost-effective opportunity. The current challenge for the field is to determine the set of genes and variants for which sufficient evidence is available to justify their examination during drug development on a routine basis.

2.4 Development of an ADME list of Genes and Genetic Markers:

The FDA recognizes that integrated multidisciplinary groups need to be formed in order to tackle the goals set out in the Critical Path. These goals are not achievable by one single company, university or government agency alone[236]. The initiative described herein, to create a consensus "Core List" of ADME genes was launched by the pharmaceutical industry because it became clear that there was a need to develop a standardized list of "evidence based" genes with enabling technological tools to support drug development. Along these lines, a unique partnering of a group of representatives from the pharmaceutical industry and an academic center came together to develop a consensus list of ADME genes and genetic variants that would be used as an initial tool to better understand drug disposition. The objective of the partnership was to assemble a diverse group of researchers and develop a consensus "Core List" of ADME genes and markers that could be employed in the drug development process, in support of FDA submissions, and ultimately in general clinical practice. Representatives from the following pharmaceutical companies participated in this effort: GlaxoSmithKline, Research Triangle Park, NC, USA; Eli Lilly and Company, Indianapolis, IN, USA; Abbott Laboratories, Abbott Park, IL, USA; Johnson & Johnson, Raritan, NJ, USA; Bristol-Myers Squibb, Lawrenceville, NJ, USA; F. Hoffmann-La Roche, Basel, Switzerland; Sanofi-Aventis, Bridgewater, NJ, USA.; Merck & Co Inc., Whitehouse Station, NJ. USA. Whithin these organizations, representatives from with

backgrounds in pharmacogenomics, biomarkers, drug development, and molecular diagnostics provided input to the final ADME content.

The initiative was divided into four distinct phases: characterization and compilation of an ADME gene list, criteria and selection of ADME genes for a Core List, criteria and selection of genetic variants for the core gene list, and development of an extended list of ADME and ADME-related genes.

2.4.1 Phase I: Characterization and Compilation of an ADME Gene List

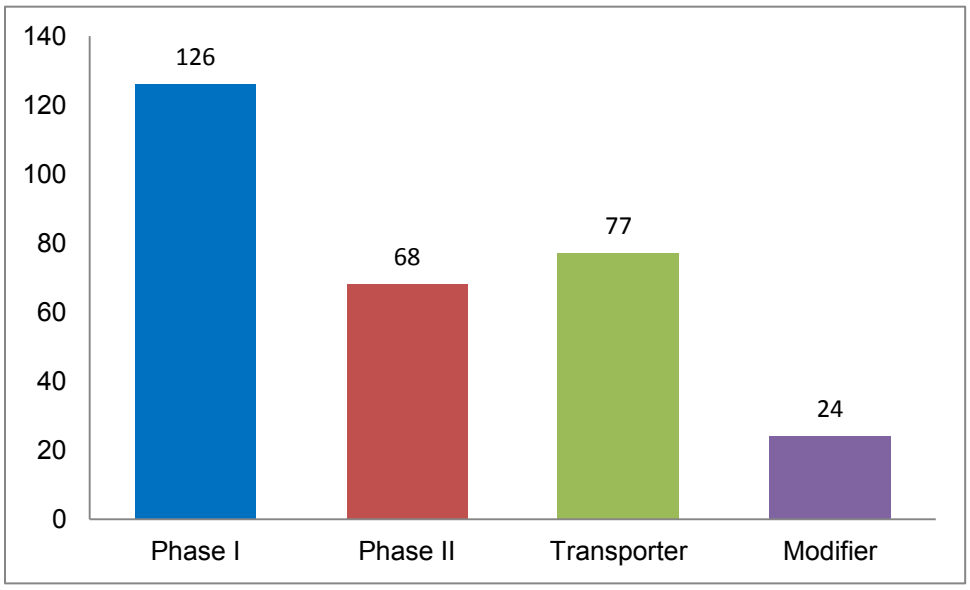
The first step of the process was to build a global list of genes known or thought to be involved with metabolism and transport of pharmaceuticals. The approach was to ask each participating organization to devise a list of genes of interest to them and submit their list to a central organizer. A total of 9 lists (from pharmaceutical companies and an academic center) were received and the number of genes per list varied from as few as 10 to as many as 350. One of the first major challenges encountered during this exercise was the merging and consolidation of the genes lists, which need to be converted into a single standardized format. This included mapping all genes to a unique chromosomal position on the current build of the human genome (NCBI 36.1), using the correct HUGO (www.genenames.org) nomenclature to describe genes, and using the same naming scheme to define genetic variants. Once the lists were identically formatted, it was discovered that a few genes were common to most lists, but that many genes were unique to one or two lists. This clearly illustrated some of the challenges that were to be faced by the group in establishing consensus on a relevant core set of genes.

Once the lists were consolidated, genes were grouped into four categories: 1) phase 1 enzymes, responsible for oxidation/reduction reactions; 2) phase 2 enzymes, responsible for the

conjugation reactions; 3) transporters, responsible for the movement of drugs in and out of tissues or compartments; and 4) drug metabolism modifiers, that can either alter the expression of, or affect the biochemistry of, ADME genes. As not all the genes on the initial nine lists fell into these four categories, a second grouping was identified as *ADME-related genes;* this list consisted of genes involved in pharmacodynamic mechanisms. These are genes identified as being drug targets, receptors, ion channels or genes involved in individual drug effect mechanisms. While many of these genes are important for a pharmacological response of a specific drug, they are not broadly applicable across many therapeutic classes of medication. For example a gene that was common to many of the participants' lists but that was characterized by the group as an ADME-related gene was the gene encoding catechol-o-methyltransferase (COMT) which is part of the degradative pathways for catecholamine transmitters but did not fulfill the required criteria of a "drug metabolizing"[237].

The group decided that genes involved in disease predisposition and prognosis were ineligible due to the ethical and legal considerations involved in screening for these variations. For example, variations in glucose-6-phosphate dehydrogenase (G6PD) can predispose individuals to hemolytic anemia. Thus, even though G6PD can influence a patient's response to 6-mercaptopurine, it was none-the-less dropped from the list for potential ethical and legal reasons[238]. After normalization of the information contained within the individual lists and application of the criteria described above, a final master list of 295 ADME genes was obtained (Figure 12)**.** It is composed of 126 genes categorized as phase I enzymes (43%); 68 genes categorized as phase II enzymes (23%); 77 genes categorized as transporters (26%); and 24 genes identified as ADME gene modifiers (8%).

Figure 12: Composition of the Complete ADME List of Genes.



A total of 295 genes were deemed to be directly involved in drug metabolism and/or had the ability to influence a drug's pharmacokinetic profile. Of the four categories that the genes were placed in: 126 (43%) where categorized as phase I enzymes (Blue); 68 (23%) where categorized as phase II metabolic enzymes (Red); 77 (26%) where categorized as transporters (Green); and 24 (8%) where identified as drug metabolism modifiers (Purple).

2.4.2 Phase II: Criteria and Selection of Genes for a Core List

Although it would be of interest to sequence the comprehensive list of ADME-relevant genes in every patient, analyzing, classifying and housing the data would be an impossible task in addition to the current prohibitive cost of sequencing at this level. Therefore, the group decided that a key deliverable of their collaborative efforts would be an ADME Core List, cataloging those genes deemed most justifiable to include on a screening panel for routine use. Thus, genes in the "Core List" were selected to fit one or more of the following criteria: 1) genes listed as US-FDA validated;[239] 2) genes which had a significant body of published evidence in the literature indicating the gene or its variants were involved in drug disposition; or 3) key

opinion leaders had evidence that the gene or its variants were involved in drug disposition. The goal here was not to be overly restrictive, but to get a consensus list of important or 'core' genes. It was acknowledged by the group that as more data is generated, the number of genes on the Core List may be greater or lesser to reflect the evolving state of our knowledge of drug metabolism and excretion.

Table 6: Inclusion/Exclusion Criteria for the ADME genes and markers

- **Must be involved in drug pharmacokinetics**
- **Flagged by the FDA as validated**
- **Supported by published literature of more than one group that the variation alters gene function**
- **Supported by key opinion leaders in the drug metabolism field as altering gene function**
- **Genes associated with disease have been excluded for ethical reasons**

Using the defined set of inclusion criteria applied to the genes in Table 6, a consensus list of 32 Core ADME genes was identified (Table 7). Examples of genes that made the Core List as US-FDA validated markers included CYP2C9, CYP2C19, CYP2D6, DPYD, NAT2, TPMT and UGT1A1. Examples of genes that had an extensive body of supporting published evidence were CYP1A1, CYP1A2, CYP2B6, NAT1, SULT1A1, ABCB1 (MDR1) and SLCO1B1 (OATPC). Other genes that were strongly recommended by experts working in pharmacokinetics and drug development included genes like CYP3A7 and the GST's.

Table 7: Gene Composition of the ADME Core List

Gene Symbol	Full Gene Name	Class
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	Phase I
CYP1A2	cytochrome P450, family 1, subfamily A, polypeptide 2	Phase I
CYP2A6	cytochrome P450, family 2, subfamily A, polypeptide 6	Phase I
CYP2B6	cytochrome P450, family 2, subfamily B, polypeptide 6	Phase I
CYP2C19	cytochrome P450, family 2, subfamily C, polypeptide 19	Phase I
CYP2C8	cytochrome P450, family 2, subfamily C, polypeptide 8	Phase I

CYP2C9	cytochrome P450, family 2, subfamily C, polypeptide 9	Phase I
CYP2D6	cytochrome P450, family 2, subfamily D, polypeptide 6	Phase I
CYP2E1	cytochrome P450, family 2, subfamily E, polypeptide 1	Phase I
CYP3A4	cytochrome P450, family 3, subfamily A, polypeptide 4	Phase I
CYP3A5	cytochrome P450, family 3, subfamily A, polypeptide 5	Phase I
DPYD	dihydropyrimidine dehydrogenase	Phase I
GSTM1	glutathione S-transferase M1	Phase II
GSTP1	glutathione S-transferase pi	Phase II
GSTT1	glutathione S-transferase theta 1	Phase II
NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)	Phase II
NAT2	N-acetyltransferase 2 (arylamine N-acetyltransferase)	Phase II
SULT1A1	sulfotransferase family, cytosolic, 1A, member 1	Phase II
TPMT	thiopurine S-methyltransferase,	Phase II
UGT1A1	UDP glucuronosyltransferase 1 family, polypeptide A1	Phase II
UGT2B15	UDP glucuronosyltransferase 2 family, polypeptide B15	Phase II
UGT2B17	UDP glucuronosyltransferase 2 family, polypeptide B17	Phase II
UGT2B7	UDP glucuronosyltransferase 2 family, polypeptide B7	Phase II
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	Transporter
ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	Transporter
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	Transporter
SLC15A2	solute carrier family 15 (H+/peptide transporter), member 2	Transporter
SLC22A1	solute carrier family 22 (organic cation transporter), member 1	Transporter
SLC22A2	solute carrier family 22 (organic cation transporter), member 2	Transporter
SLC22A6	solute carrier family 22 (organic anion transporter), member 6	Transporter
SLCO1B1	solute carrier organic anion transporter family, member 1B1	Transporter
SLCO1B3	solute carrier organic anion transporter family, member 1B3	Transporter

In order to facilitate data entry and maximize participant input, a website was created to manage the project (www.PharmaADME.org). The website is publicly available and contains all the gene and marker information for the ADME Core List, as well as details on the inclusion criteria established by the group. Further, the website allows users to provide feedback and suggestions on additional genes or genetic variants for inclusion on the Core List. It is important to note that the gene and marker lists are not static. The lists will be periodically updated as the body of knowledge pertaining to ADME-related genes develops.

2.4.3 Phase III: Criteria and Selection of Genetic Variants on Core Gene List

Once the Core ADME Gene List of 32 genes was established, an accompanying set of genetic variants, or markers, within these genes needed to be created, since this known variation is what would be accessed in patient samples. Criteria for inclusion of the markers mirrored what the group had established for gene selection. Thus, variants had to be: 1) US-FDA validated variants; 2) supported by published literature from more than one group that the variation altered gene function; 3) supported by key opinion leaders in the drug metabolism field as altering gene function; or 4) one that causes an amino acid change in the protein encoded by the gene.

In parallel with the gene lists, each of the participating groups submitted lists of genetic variants for their list of ADME genes. Marker identification and nomenclature assignment was complicated by the fact that many of the markers were not included in public databases, such as dbSNP, and relevant genetic variants were comprised of not only Single Nucleotide Polymorphisms (SNPs), but also insertions and deletions (InDels), and copy number variants (CNVs). Therefore, to enable the merging of the lists, a defined set of required information was established for each maker including at minimum: chromosome number, chromosomal position, variation at that position, and upstream and downstream flanking sequences (~50 bp). Having this information was also necessary to remove redundancy from the marker list.

Markers were passed through a quality control (QC) process to verify their sequence and location by aligning the flanking sequences of the variant to the human genome using Blat (genome.ucsc.edu). This process was straight forward for those markers that were in present in curated databases such as dbSNP and Seattle SNPs (pga.gs.washington.edu). For rare functional markers and non-SNP variations such as CNVs, that were not present in the above mentioned databases, this process of QC was much more difficult. In most instances, it was necessary to

retrieve the original publication for the variant to validate the information. Often inferring sequence information or retrieving PCR primer designs from the original publications was necessary to ascertain the relevant information. This QC process was critical to standardize the information contained in the core variant list.

Another challenge identified in assembling the marker list was the use of the star (*) nomenclature for genes where it is the established naming scheme, the standard used for the cytochrome P450s[131]. The difficulty with this type of naming scheme is that this nomenclature often defines haplotypes, and while many of these haplotypes are defined by a single marker, in some cases they are not. Although this information is relevant, its usefulness is derived by its presence in a defined haplotype and often requires translation tables to define a specific star phenotype from a variable set of genotypes. The group thoroughly reviewed the literature and websites to carefully define the minimal markers required to accurately define all star genotypes that were selected for the ADME core marker list (see www.PharmaADME.org).

To help further characterize the markers, the group placed all the markers into one of 3 categories: 1) validated functional markers, 2) potential functional markers and 3) investigative markers. As with the gene lists, criteria were established for a marker to be considered a validated functional marker (see Table 1). Those markers that met some but not all criteria were considered potential functional markers and the remainder were classified investigative. Furthermore, the group agreed that all haplotype tagging markers, that did not meet the above criteria would be removed from Core List because their clinical value is unclear.

Following the inclusion criteria for genes and markers, an agreed upon consensus list of 184 markers for the 32 genes comprises the Core ADME Variant List (Table 8). A complete list

of information defining the marker's chromosomal number, chromosomal position, variation, and flanking sequence information can be found at www.PharmaADME.org.

Table 8: Core ADME Marker List

Gene	Markers
CYP1A1	***2A, *4, *3, *5, *8, *6, *7**
CYP1A2	***1K, *1C, *1F, *7**
CYP2A6	***2, *9 , *11, *17, *8, *6, *7, *5, *12, *1X2a, *1X2b, *20, *4**
CYP2B6	***8, *16, *28, *6, *4, *2**
CYP2C19	***4, *17, *8, *2, *3, *6, *7, *12, *5**
CYP2C8	***3, *4, *3, *2, *5, *7, *8**
CYP2C9	***3, *2, *9, *11, *5, *8, *10, *6, *12, *13, *15, *25, *4**
CYP2D6	***10, *2a, *2, *15 , *17, *41, *4, *6, *12, *11, *7, *3, *14, *8, *9, *18, *19, *20, *21, *38, *40, *42, *44, *5, *56**
CYP2E1	***2**
CYP3A4	***6, *2, *20**
CYP3A5	***6, *10, *3, *5, *7**
DPYD	***9A, *8, *9B, *10, *7**
GSTM1	***B , *X2, Null**
GSTP1	**V114A, V105I**
GSTT1	**Null**
NAT1	***14, *11 , *15 , *19 , *17 , *22, *5**
NAT2	***12, *6, *7, *5, *13, *11, *14 , *19**
SULT1A1	***3, *2, *4, Null, XN**
TPMT	***3c, *3b, *2 , *4, *8, *3a**
UGT1A1	***7, *27, *60, *6, *28 _ *36 _*37, *29**
UGT2B15	***2**
UGT2B17	***2**
UGT2B7	***2**
ABCB1	**3435C>T, T1236T>C, 2677T>G,A, -129T>C**
ABCC2	**V417I, I1324I, -24C>T, R768W, S789F, 1450A>T**
ABCG2	**421C>A, Q126X**
SLC15A2	**P409S, R509K, L350F, A284A**
SLC22A1	**R61C, P341L, G465R, G220V, P283L, R287G, M420del, M408V, C88R, G401S**
SLC22A2	**S270A, M165I, R400C, K432QP54S**
SLC22A6	**R50H**
SLCO1B1	***1B, *5, *10, *11, *12, *2, *3, *3, *6, *9**
SLCO1B3	**334T>G , 699G>A**

2.4.4 Phase IV: Development of an Extended List of ADME and ADME Related Genes

Genes that were not included in the Core ADME list, but listed as important to drug metabolism or ADME by at least one participant where included in a list identified as the ADME Extended list. The final Extended ADME List has 263 genes (Table 9). The list is comprised of the following: 114 phase 1 metabolizing enzymes, 57 phase 2 metabolizing enzymes, 68 transporters and 24 ADME modifiers.

Due to the large number of genes present in the Extended ADME gene list, a decision was made to have each of the participants prioritize and rank each of the genes within the list. The question posed to the participants was, "Would you be interested in including this ADME gene in a set of markers to be used to screen your clinical populations?" Using the www.PharmaADME.org web interface, all genes were defaulted as selected, so participants had to log-in and individually deselect genes for non-inclusion. All but one company participated in this exercise. Seven of the members participated in the ranking of the Extended ADME genes. Therefore the highest rank a gene could receive was 7. Forty-three (16%) of the genes on the list where given a rank of 7 (Table 9). A complete set of gene information for the Extended List can be found at the website, www.PharmaADME.org. This list is also not static and will be re-evaluated on a regular basis in order to reflect evolving functional data.

Many of the original 399 genes submitted were of importance to various participants but which were not considered to be involved directly in drug metabolism or ADME. A list of 76 genes comprises the ADME-related gene list. Some examples of genes on this list are catechol-O-methyltransferase (COMT), as outlined above, but others include the vitamin K epoxide reductase complex, subunit 1 (VKORC1) involved in the pharmacodynamics of warfarin and

monoamine oxidase (MAO) which is the target of MAO inhibitors[240–242]. A similar ranking exercise was performed on the ADME-related List as was done with the ADME Extended List, however for this panel, as a default, the genes were not selected and a participant had to actively select a gene for it to be counted. Of the 76 genes on the ADME-related List, only 2 genes had a rank of 4 (MAO-A and MAO-B) and a total of 20 (26%) had a ranking of between 1 and 4. All the data on the ADME-related List can be found at www.PharmaADME.org.

Table 9: Genes (with rank*) of the Extended List

Rank	Gene	Full Name	Type
7	ALDH4A1	aldehyde dehydrogenase 4 family, member A1	Phase I
7	EPHX1	epoxide hydrolase 1, microsomal (xenobiotic)	Phase I
7	FMO3	flavin containing monooxygenase 3	Phase I
7	ALDH5A1	aldehyde dehydrogenase 5 family, member A1	Phase I
7	CYP7A1	cytochrome P450, family 7, subfamily A, polypeptide 1	Phase I
7	CES1	carboxylesterase 1 (monocyte/macrophage serine esterase 1)	Phase I
7	CES2	carboxylesterase 2 (intestine, liver)	Phase I
7	ALDH6A1	aldehyde dehydrogenase 6 family, member A1	Phase I
7	GSTM2	glutathione S-transferase M2 (muscle),glutathione S-transferase M4	Phase II
7	GSTM3	glutathione S-transferase M3 (brain)	Phase II
7	GSTM4	glutathione S-transferase M4	Phase II
7	UGT1A3	UDP glucuronosyltransferase 1 family, polypeptide A3	Phase II
7	UGT1A6	UDP glucuronosyltransferase 1 family, polypeptide A6	Phase II
7	UGT1A7	UDP glucuronosyltransferase 1 family, polypeptide A7	Phase II
7	UGT1A8	UDP glucuronosyltransferase 1 family, polypeptide A8	Phase II
7	UGT1A9	UDP glucuronosyltransferase 1 family, polypeptide A9	Phase II
7	SULT1B1	sulfotransferase family, cytosolic, 1B, member 1	Phase II
7	UGT2A1	UDP glucuronosyltransferase 2 family, polypeptide A1	Phase II
7	UGT2B11	UDP glucuronosyltransferase 2 family, polypeptide B11	Phase II
7	UGT2B28	UDP glucuronosyltransferase 2 family, polypeptide B28	Phase II
7	UGT2B4	UDP glucuronosyltransferase 2 family, polypeptide B4	Phase II
7	GSTA1	glutathione S-transferase A1	Phase II
7	GSTA2	glutathione S-transferase A2	Phase II
7	GSTA3	glutathione S-transferase A3	Phase II
7	GSTA4	glutathione S-transferase A4	Phase II

7	GSTA5	glutathione S-transferase A5	Phase II
7	GSTO1	glutathione S-transferase omega 1,glutathione S-transferase omega 2	Phase II
7	GSTO2	glutathione S-transferase omega 2	Phase II
7	SULT1A2	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 2	Phase II
7	SULT1A3	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 3	Phase II
7	GSTT2	glutathione S-transferase theta 2	Phase II
7	ABCB8	ATP-binding cassette, sub-family B (MDR/TAP), member 8	Transporter
7	SLC22A11	solute carrier family 22 (organic anion/cation transporter), member 11	Transporter
7	SLC22A8	solute carrier family 22 (organic anion transporter), member 8	Transporter
7	SLCO2B1	solute carrier organic anion transporter family, member 2B1	Transporter
7	SLCO1A2	solute carrier organic anion transporter family, member 1A2	Transporter
7	ABCC4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	Transporter
7	SLC15A1	solute carrier family 15 (oligopeptide transporter), member 1	Transporter
7	SLC10A1	solute carrier family 10 (sodium/bile acid cotransporter family), member 1	Transporter
7	ABCC12	ATP-binding cassette, sub-family C (CFTR/MRP), member 12	Transporter
7	SLC7A5	solute carrier family 7 (cationic amino acid transporter,), member 5	Transporter
7	ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	Transporter
7	AHR	aryl hydrocarbon receptor	Modifier

*Table 4 contains only the 43 genes of the extended list that had the highest rank of 7 out of 7. The remainder of the list can be found on www.PharmaADME.org. The gens are divided into four classes: phase I metabolizing enzymes (Blue), phase II metabolic enzymes (Red), transporters (Green), and ADME modifiers (Purple).

2.5 Discussion:

The process of defining a Core List of ADME gene was developed as a successful collaboration between pharmaceutical companies and an academic center. The result is an industry directed evidence-based consensus Core List of ADME genes that has been well vetted and agreed upon by all participants. Remarkably, despite very different directed research programs, the diverse group was able to come to agreement on a concrete set of genes using a pre-defined set of criteria. This ADME Core List will now form the basis for a minimal set of genes and related markers that could be used to screen clinical populations involved in drug development by the agreed parties. As a second objective for the ADME Core List, the group also had the goal of using the list to provide a framework for the various technology platform providers to create standardized products that will be broadly applicable across many therapeutic classes of drugs. This has been accomplished with the development of three commercially available assays: the Affymetrix DMET Plus Premier Pack [243–245] , the Sequenom iPLEX® ADME PGx panel[246] and the Illumina VeraCode ADME Core Panel[247]. While the VeraCode product consists solely of markers on the Core List, the DMET chip and iPLEX ADME PGx panel contain additional markers in genes from the extended ADME list (Table 10).

Table 10: Contents of Three Commercially Available ADME Genotyping Products Based on Content Derived by the PharmaADME Working Group.

Company	Assay	Number of Genes	Number of Markers	Design Criteria
Affymetrix®	DMET™ Plus Premier Pack	225	1936	90 % of ADME core list plus additional putative functional variants in other ADME genes.
Sequenom®	iPLEX® ADME PGx panel	36	192	99 % of ADME core list plus additional functional haplotypes
Illumina®	VeraCode ADME	34	184	Known functional variants

Core Panel	including deletion and duplications in ADME genes as defined by the ADME Core List

While the ADME Core List can be applied to drug development in several ways, it was not conceived to be only limited to drug development uses. The group intended the list to be used in any application where knowledge of variability in metabolism or transport could aid decision making. Public access to the lists through www.PharmaADME.org and the sharing of the selection criteria was deemed the most reliable way for dissemination to the broadest audience.

In generating the ADME Core List, which contained the genes with documented effect on drug disposition, the group also compiled a list of additional ADME genes, the ADME Extended list, which does not have the same evidence base as the ADME Core List. It is reasonable to assume that some of the genes in this extended list will ultimately be found to have a significant role in the metabolism or transport of new or existing compounds. Thus, both the Core and Extended Lists are to be updated on a regular basis.

Throughout the process of defining the ADME gene and variant lists, several prescribed uses became evident as to how this information will be used in practice. The two predominant models are the evidence based approach and the broader hypothesis free screening approach. In the evidence based approach only a limited group of genes, the ADME Core list, would be useful. This would be the case for pharmaceutical driven clinical studies because this list was founded on compiled, known functional evidence. The purpose of the Core List was to have an agreed upon list of variants, summarizing all current knowledge, that would be useful for evidence based decision making in clinical trials. In the hypothesis free screening approach the

Extended List would be more useful, where it would be necessary to cast a broader, more inclusive net for additional genes potentially involved in metabolism or transport to identify correlations between genotype and observed outcomes of the trial. An added rationale for using the broader ADME list was to enable the identification of new pathways that might be involved in the metabolism of a compound of interest where the technology was not previously available. A good example of the latter design was the recent finding of the involvement of CYP4F2 in warfarin metabolism.[248]

To address the evolution of our knowledge with respect to variation in drug metabolism pathways, the group has agreed to meet on an annual or bi-annual basis to review, update and discuss the information in the respective lists. The www.PharmaADME.org website is intended to be a public portal for this information. It is important to note that the gene and marker lists are thought of as moving targets. Hence, the lists will be updated as the body of knowledge pertaining to ADME continues to grow. The website is also intended to be a point of access where individual researchers can make suggestions for the inclusion of additional genes and variants to either list.

The information established by this unique collaboration of academic and pharmaceutical partners could be used as a working model for future collaborations with the purpose of addressing the FDA's Critical Path initiative. Efforts like this can only help advance the integration of genomic biomarkers into the drug development process and clinical practice. As new research in the areas of drug efficacy and toxicity continues, the prioritization of genes and markers will evolve. Nonetheless, the necessary framework has been established to incorporate new knowledge. In the future, we anticipate the participation of even more parties who will share the same goal of applying genetics to improving patient outcomes.

3 The Development of a Broad Based ADME Panel for use in Pharmacogenomic Studies

This section describes the development and testing of a broad based ADME genotyping assay. The assay has gone through three design iterations where over 27 000 oligonucleotides have been tested to interrogate functional and tag SNPs in ADME genes. The assay was developed to address the gap in commercially available ADME genotyping assays that either contain a small number of functional variants or do not have sufficient coverage of ADME genes. Genes and markers for the genotyping panel were selected in collaboration with many groups from both academia and the pharmaceutical industry in an effort to capture all pertinent genes and metabolic pathways that have been implicated in drug metabolism. The final assay design was composed of just over 3,000 markers in 181 genes. Over three phases of iterative development, the assay conversion rate for the 3,000 markers was improved from 83% to 97.4% through the incorporation of novel design strategies to overcome areas of genomic interference such as regions of homology and underlying polymorphisms. Accuracy of the assay was validated by screening more than 200 samples of known genotype with a concordance >98%. Additionally, data from the assay has also been compared to data from different technological platforms and has an overall concordance of >99.5%. The effectiveness of the design strategy was demonstrated in the successful utilization of the assay in the screening of over 1000 samples in various pharmacogenomic studies. Of particular interest are the novel associations with ADME gene variation and two different adverse drug reactions in children uncovered using the assay.

The Development of a Broad Based ADME Panel for use in Pharmacogenomic Studies

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3.1 Abstract

In order to optimally address the inter-individual variability observed in pharmacokinetic drug response, we have created a custom genotyping panel that interrogates most of the key genetic variations present in a set of 181 prioritized genes responsible for the Absorption, Distribution, Metabolism and Excretion (ADME) of many therapeutic agents. This consensus list of genes and variants was based on the ADME Core and Extended gene lists compiled by a group of Pharmaceutical companies as having relevance (PharmaADME.org). These pharmacokinetic genes and pathways are well known; however there has been little effort to develop tools that can interrogate a large number of these genes simultaneously within a single experiment. Using novel technical design strategies, an optimized and validated ADME genotyping panel, encompassing ~3,000 variants, has been developed that has broad applicability to any study or clinical trial that would benefit from the evaluation of an extensive list of ADME genes.

KEY Words: pharmacogenomics, drug metabolism, ADME, genotyping, SNPs

3.2 Introduction

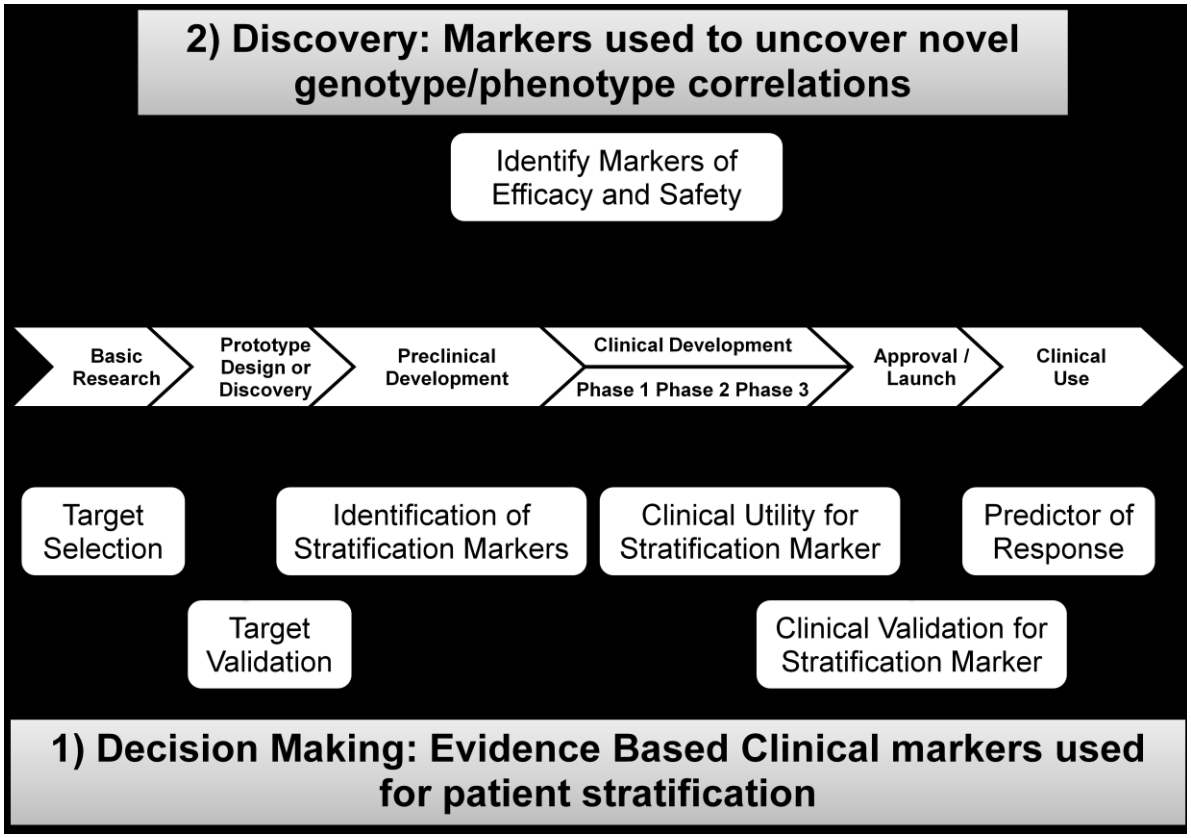
Many medications show clear patient variability in terms of efficacy and undesired adverse side effects[249,250]. This variability can have significant impact on medical outcomes and the success or failure of drug development programs. While a portion of this variability can be attributed to patient characteristics such as age, sex and disease state, a substantial amount of the variability in drug response in patients is due to genetic variation[250]. Genetic variation in drug response can be divided into two major areas pharmacodynamics (PD) and pharmacokinetics (PK). Pharmacodynamics involves the interaction of the drug with its target and is directly responsible for a patient's pharmacologic dose dependent response[4]. Pharmakinetics, or drug metabolism, determines the amount of the active drug that is available to interact with its target and for how long it remains active in the body[251]. There is a substantial amount of literature outlining a well understood set of genes and pathways responsible for the metabolism and mechanisms of action for many drugs[73,252–254]. Of particular relevance are the PK pharmacogenetic biomarkers that are responsible for the Absorption, Distribution, Metabolism and Excretion (ADME) of drugs and their metabolites. PK genes are applicable to almost all medical compounds; while PD genes are primarily involved with a specific drug target and are most often only specific to a particular medical compound and its mechanism of action. Thus, when developing tools to help integrate genetics into healthcare and drug development programs, a focused PK derived pharmacogenetic biomarker tool will have greater utility and application across many therapeutic areas than a set of PD derived genetic biomarkers which will only be applicable to a single medical compound or class of drugs. While the concept of applying pharmacogenetics to the drug development process has been debated for over a decade, consensus on exactly how it can be applied is still being developed[255].

Genetic variation observed in ADME genes can be grouped into two categories; functional and those having unknown functional consequence. Evidence based biomarkers, or known functional variants, have been experimentally demonstrated to alter drug response[239]. Only with evidence based/functional biomarkers can a physician make actionable decisions in the treatment of patients. Moreover, only functional biomarkers can be used to help stratify pharmaceutical clinical trials to improve efficacy and safety profiles[256,257]. For example, several CYP2C19 based biomarkers/variants have been shown to reduce CYP2C19 activity which is necessary for the activation of the drug clopidogrel (Plavix) in both retrospective and prospective studies[171–175]. Thus, patients possessing loss of function polymorphisms in the CYP2C19 gene will have reduced efficacy of Plavix and be at a higher risk of thrombolytic events. These types of well documented observations are now becoming routinely incorporated into the labels of many drugs by the FDA [239]. If an ADME genetic variation has yet to be functionally characterized, it falls into the category of variations with unknown functional consequence. Theses hypothesis generating variants have the potential to be functional but must first be tested for association with drug response in well phenotyped patients. An example of this type of variation includes the variants in TPMT and COMT that have recently been identified and associated with ototoxicity in children receiving cisplatin[258]. This observation is presently being validated in additional patient populations.

The full implication of the influence of genetic variation on drug response is presently poorly understood for many drugs and requires a broader investigation of both PK and PD pathways; however, a tool that interrogates evidence based and hypothesis generating variation in ADME genes would have significant application to clinical trials involving any medication. Such a tool could be used to identify metabolism status phenotypes, to assist with patient

decision making either as: a covariate in a statistical analysis, exclusion criteria in a clinical trial, a predictor of drug response and toxicity, or as guidance of dose selection. Moreover, when there appears to be no evidence for functional variants affecting response, haplotype tagging variants in ADME genes can be used to uncover new correlations between response or toxicity and genetic variation (see Figure 13). It is for these reasons that we set out to create a genotyping assay that not only includes known functional variation in ADME genes, but also encompasses tag SNPs that account for blocks of Linkage Disequilibrium (LD) across many ADME genes. Such an assay will have application for both the evidence based functional characterization of patient populations as well as have the ability to identify and discover new genetic biomarkers in ADME genes.

Figure 13: The Uses of ADME Pharmacogenomics in Research and Drug Development



The design of the ADME panel allows for two different yet complimentary applications. 1) Validated functional variants able to identify metabolism status phenotypes that can assist with patient decision making either as: a covariate in a statistical analysis, exclusion criteria in a clinical trial, a predictor of drug response and toxicity, or as guidance of dose selection. 2) Haplotype tagging variants in ADME genes can be used to uncover new correlations between response or toxicity and genetic variation.

3.3 Selection of the Genotyping Platform

As outlined above, there is a need for a comprehensive ADME panel that can query both functional and tagging SNPs. To address this deficiency, we have embarked on developing an optimized and validated broad based ADME panel that can be used to screen a significant

number of drug metabolism (PK pathway) genes for use in pharmacogenomic studies. A genotyping tool of this nature would be complimentary to most currently available commercial ADME genotyping assays that are primarily focused on a small number genes and functional variants[243,246,247,259–262] (see Table 11). Moreover, whole genome SNP arrays often lack key markers in most ADME loci due to complexities in theses genomic regions and the tagging SNP coverage of ADME genes is often poor in these commercial products (see Table 12)[263]. Recently, it was demonstrated that even whole genome arrays with more than 2.5 million SNPs still cover no more than 85% of 253 ADME genes[264]. There have been many pharmacogenomic genome wide association studies (GWAS) and save a few examples [202–210], most have been largely unsuccessful in identifying genes that are predictive of response or toxicity[201]. Interestingly a number pharmacogenomic GWAS studies failed to identify significant associations in genes that were not already known from previous ADME candidate gene studies[202–210].

Table 11: Content of Commercially Available ADME Genotyping Products Compared too Developed Panel.

Company	Assay	Number of Genes	Number of Markers	Design Criteria
NA	ADME Panel	~180	3000 (plus the ability to spike in an additional 72)	known and putative functional variation in ADME genes as well as tag SNPs that account for blocks of Linkage Disequilibrium (LD) across ADME genes
Affymetrix®	DMET™ Plus Premier Pack	225	1936	Known functional and putative functional variants in ADME genes.
Sequenom®	iPLEX® ADME PGx panel	36	192	99 % of ADME core list plus additional functional haplotypes
Illumina®	VeraCode ADME Core Panel	34	184	Known functional variants including deletion and duplications in ADME genes as

				defined by the PharmaADME working group
Roche®	AmpliChip™ CYP450 Test	2	~22	Known functional variants including deletion and duplications in CYP2D6 and CYP2C19
Progenika®	PHARMAchip	36	~90	Known functional variants including deletion and duplications in PK and PD genes.

Table 12: Content Unique to the ADME Panel in Comparison to Several Commercially Available Assays.

Assay:	**Affymetrix DMET plus**	**Affymetrix 500K**	**Illumina HumanHap550**	**Affymetrix 6.0**	**Illumina OmniQuad**
Number of SNPs	1936	500,000	550,000	907,000	1,000,000
ADME Panel SNPs Included on Assay	225	405	1038	1017	2088
ADME Proxy (r2 >0.8)	66	1446	495	702	168
Unique to ADME Panel Content	**90.3% (2709 SNPs)**	**38.3% (1149 SNPs)**	**48.9% (1467 SNPs)**	**42.7% (1281 SNPs)**	**24.8% (744 SNPs)**

For the development of this ADME genotyping tool, Illumina's GoldenGate genotyping technology was selected as the preferred platform for its multiplexing capabilities (up to 1536 SNPs in a single oligo pool array (OPA)), high throughput capability (96 samples in a single experiment), and ease of use[219]. An additional advantage of the Illumina GoldenGate technology versus other genotyping technologies is its proven consistency and accuracy. GoldenGate chemistry involves three levels of specificity; independent hybridization, primer extension and ligation steps all combine to increase the precision of allele discrimination of the assay. Furthermore, highly reproducible results are achieved because each genotyping data point is a

result of an average of ~30 independent measures[219]. A further benefit of selecting the GoldenGate chemistry for this development was that this was the primary technology used to generate much of the HapMap data and successful validated designs were already available for many of the selected SNPs[101].

3.4 ADME Content: The PharmADME Core List

An industry initiated effort was launched to develop a consensus, "Core List" of standardized "evidence based" drug metabolizing (ADME) genetic biomarkers that are broadly applicable to many pharmaceutical clinical trials and FDA drug submissions. The effort was driven by a unique multidisciplinary group of representatives from the pharmaceutical industry (see section 2.4). Our goal was to develop a list of genes and genetic biomarkers that could be screened using today's technology platforms to identify predictors of pharmacokinetic variability that could impact drug safety and efficacy in the current drug development process. Through a phased process and using pre-defined criteria, we identified a "Core List" of 32 ADME genes, which includes 184 markers that can be used to screen subjects in clinical trials (see section 2). Thus, genes and markers in the "Core List" were selected to fit one or more of the following criteria: 1) genes listed as US-FDA validated;[239] 2) genes which had a significant body of published evidence in the literature indicating the gene or its variants were involved in drug disposition; or 3) key opinion leaders had evidence that the gene or its variants were involved in drug disposition. The goal here was not to be overly restrictive, but to get a consensus list of important or 'core' genes. Additionally, we ranked a further 300 ADME genes that are likely involved in a patient's pharmacokinetic response but have not been thoroughly investigated yet.

The ADME Core genes can be grouped into four categories: Phase I drug metabolism enzymes, such as members of the cytochrome P450 superfamily responsible for non synthetic oxidation and reduction reactions that render drugs more polar or water soluble; phase II drug metabolism enzymes, responsible for the conjugation with endogenous moieties such as glucuronidation (uridine diphosphate-glucuronosyltransferases) or sulfonation (sulfotransferases); transporters, responsible for the uptake and excretion of drugs in and out of cells, such as the solute carrier (SLC) family; and modifiers, that can either alter the expression of ADME genes or affect the biochemistry of ADME enzymes, such as the retinoid X receptor [77].

As a second objective for the ADME Core List, the group also had the goal of using the lists to provide a framework for various technology platform providers to create standardized products that could be broadly applicable across many therapeutic classes of drugs. This has been accomplished with the development of three commercially available assays: the Affymetrix DMET Plus Premier Pack [243–245] , the Sequenom iPLEX® ADME PGx panel[246] and the Illumina VeraCode ADME Core Panel[247].

The results of this collaboration are available on the web at www.pharmaADME.org. The website is intended to be a public portal for this information and the lists will be updated as the body of knowledge pertaining to ADME continues to grow.

3.4.1 Gene and SNP Selection

With the comprehensive gene and SNP lists established for the ADME Core and Expanded gene categories, a SNP selection approach was initiated to incorporate both functional and tagging SNPs for all genes. The primary focus of the panel was to incorporate markers from

the ADME Core list and annotated functional markers from the highest ranked Expanded panel genes (~320 function SNPs were identified in the literature). Based on our strategy, the remaining SNP selection was to be filled with SNPs that tag haplotype blocks in our genes of interest. Our tagging strategy takes into consideration the entire gene locus and not just coding regions. Using data from the International HapMap project[101], SNPs were selected that account for blocks of linkage disequilibrium (LD) that span each ADME gene plus or minus 10kb of the gene. LD-Select[265] was used to generate bins of tag SNPs ($r^2 = 0.9$) for each of the three original HapMap populations (CEU,YRI and JPT/CHB). Tag SNPs were first selected that accounted for bins of LD in two or all three populations, followed by the selection of bins unique to a single population, with a priority for the CEU population, until the space limitation of the list was fulfilled. When possible, putative functional variants were prioritized and selected as the tag for a given bin. Examples of these include SNPs from the Human Cytochrome P450 Allele Nomenclature Committee website that either affect transcription, splicing, translation, posttranscriptional or posttranslational modifications or result in at least one amino acid change[220]. Our strategy was tailored to allow for the inclusion of the maximal content while respecting the limitation of having space within 2 Illumina GoldenGate OPA's for only 3,072 SNPs. The final assay designs also incorporated 50 SNPs with known minor allele frequencies that differ greatly in different populations to be used as ancestry informative markers. These ancestry informative markers are valuable for use in population stratification of study subjects. Finally, in the final assay design, some genotyping space was purposefully left "open" and not assigned a SNP for the inclusion of 72 flexible markers that could have the ability to be added/changed depending on the given needs of the project. The final assay includes 3000 variants in 181 ADME genes (see Appendix 3 Gene and SNP list).

3.5 Panel Development

The design of the broad-based ADME genotyping assay required that we develop 2 independent oligo pooled arrays (OPA's; consisting of 1536 SNPs per OPA) using an Illumina GoldenGate design. In order to make the majority of our identified SNP assays convert to functioning assays and to fully optimize the overall genotyping assay, three iterations of the GoldenGate ADME panel were necessary. During each phase of assay development, specific assay failure modes were addressed and different approaches were taken to improve the quality and robustness of the overall assay (see table 3). Data from each phase gave rise to insight into the subtleties and limitations of the technology and allowed for the development of novel strategies and modifications to the standard process to continually improve assay conversion rates. In addition to the testing and the validation of the assay on standardized DNA samples from the Coriell institute (www.coriell.org) and HapMap samples, each iteration of the ADME panel was used to genotype research samples of unknown genotype from several research studies during all phases of development. The three phases of development and summary results from the many applications are outlined in detail below.

Table 13: Objectives and Results of Each Assay Development Phase.

Development Phase	Objective	Results	%Conversion
Phase 1	▫ Benchmark Technology ▫ Evaluate assay conversion	▫ Identified three areas of genomic interference that disrupt assay conversion ▫ Identified errors in industrial process	83%
Phase 2	▫ Test strategies to improve assay conversion	▫ Validated assay design strategies ▫ Identified oligo synthesis inefficiencies	93.5%
Phase 3	▫ Finalize content and design ▫ Pool oligo synthesis	▫ Final Content and Design for use in research and clinical trials	97.4%

3.5.1 Phase 1

The primary goal of the first phase of assay development was to benchmark the technology, to determine what would and would not work. As all of the content on the ADME panel was selected for a defined purpose, it was imperative that the majority of the markers selected performed accurately and robustly. A marker was considered converted or functioning well when it has passed QC clustering using Illumina's Genome Studio software. A SNP has passed QC when its data points have consistent signal intensity and display tight clustering patterns for the homo and heterozygous calls.

The assay design for phase 1 consisted of two OPAs. One primarily composed of tag SNPs while the other contained functional and putative functional variants. An overlap of 93 SNPs between OPAs was also included in the design to access reproducibility. Preliminary testing of the phase

1 designs revealed that the tag OPA and the functional OPA had marker conversion rates of 96% and 70% respectfully. This was expected as many of the tag SNPs had been previously validated on the GoldenGate platform as part of the HapMap project and when possible, the SNP with the highest design score from Illumina's assay design tool was selected for LD bins with more than one tag SNP. Furthermore, many of the SNPs included in the functional SNP OPA were included despite having low design scores. This was done purposefully as this was a benchmarking exercise and we wanted to assess and establish failure modes for "must have" functional content.

3.5.2 Phase 2

After the analysis of the phase 1 genotyping results, two interesting observations were evident. First was the frequent appearance of unusual clustering patterns, which deviated from the usual three cluster patterns showing two homozygous and one heterozygous allele calls, and the second was that some of the SNPs duplicated in both OPA assays worked well in only one instance (see figure 3). This led to the identification of three consistent failure modes exhibited by the GoldenGate technology (see figure 13): underlying polymorphisms, falling within the regions of oligo binding which disrupt the binding affinity; SNP assays in close proximity, where adjacent oligos interfere with one another; and markers that lie within regions of homology that reduce the binding specificity of the oligos.

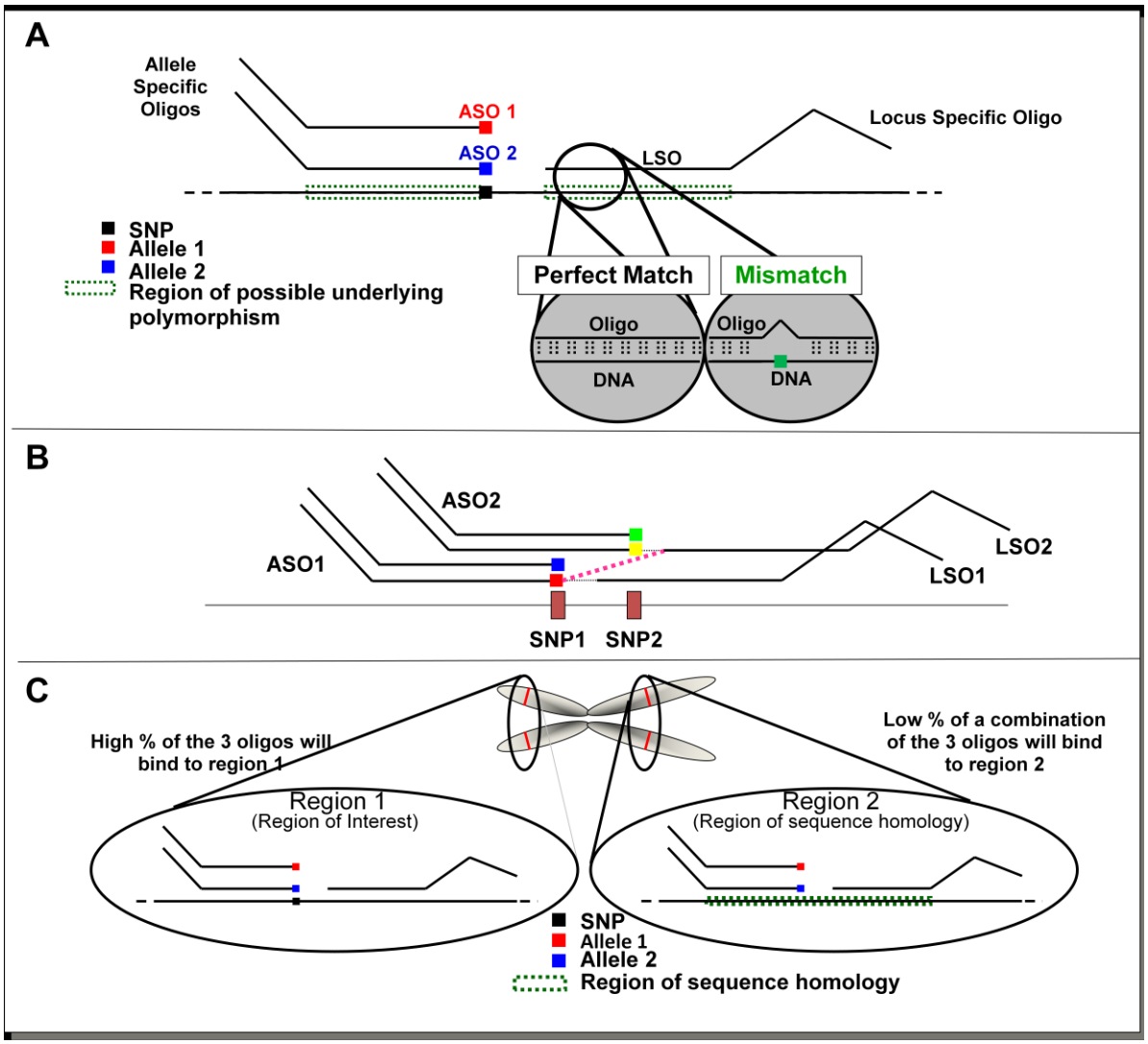
In phase 2 of development, several new design strategies were incorporated in an attempt to compensate for the above mentioned failure modes. These strategies fell outside the standard Illumina design process, and required that many of the new oligos be manually designed or synthesized separately and then added back into the OPA pools.

In order to adjust for underlying SNPs disrupting the binding affinity of oligos, degenerate bases were incorporated in the oligos. That is, oligos were designed for all possible allele combinations of the SNP being interrogated plus incorporating different combinations of the underlying SNP. In order to adjust for regions of high SNP density that generate assays that fail due to close proximity, two strategies were developed, first the distance between the allele specific and locus specific oligos was increased to limit possible interactions with adjacent oligos, however if this was not possible, we developed a method of splitting SNPs into separate OPA pools thus avoiding the interaction of adjacent oligos. The separated OPA pools could be merged back into the GoldenGate process after the binding and extension steps in order to read all markers simultaneously on a single array. Finally, in order to adjust for regions of high homology leading to a loss of specificity of the bind of the oligos, we developed a method where we could spike in PCR products in place of genomic DNA for the desired SNP containing regions of interest to increase the specificity of the assaying oligos. This strategy also allowed for specific PCR designs that were able to detect gene deletions and duplications (see Appendix 4). For example, we were able to design PCR primers that only produced a product if the CYP2D6 locus was either deleted or duplicated (see Appendix 4). Figure 14 shows successful examples of the incorporation of these strategies into the overall process recovering markers that had previously not worked. The integration of the above mentioned design strategies had the result of increasing the overall marker conversion rate from 83% to 93.5%.

As a further strategic part of the second iteration of the ADME panel, we duplicated the synthesis of one of the OPAs to test for consistency in assay conversion rates in assays that essentially should be identical. The outcome of this experiment was to assess the accuracy and consistence of the oligo synthesis process. We observed a non-overlapping SNP failure rate of

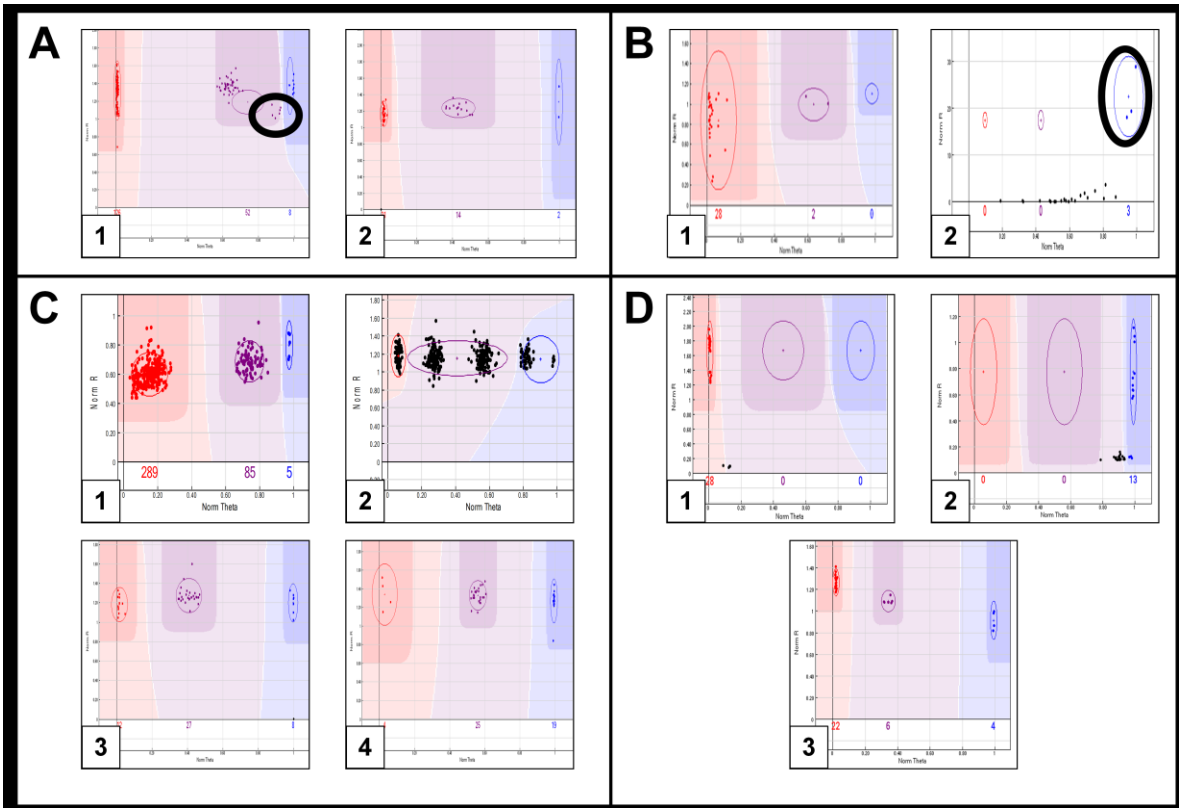
7% and 5%, respectively, in the duplicate OPA syntheses in SNPs that did not fail in the prior version of the assay. Due to the large number of oligos needed for this assay (4608 oligos per 1536 SNPs assay) it is very difficult to have 100 percent efficiency in synthesis of every oligo. In an attempt to recover these markers that were failing from apparent oligo synthesis inefficiencies, oligos from the duplicate OPA syntheses were pooled together. In the resulting pooled assay, most of the failed markers were recovered and converted successfully (see figure 14D). We observed on average 4% of markers had a pattern indicating oligo synthesis issues. In theory, if synthesis inefficiencies are random, there is a 0.16% chance that the same oligo would fail synthesis in two separate syntheses. This would indicate that we would still expect to observe 7 or 8 SNPs per 1536 SNP OPA to have synthesis issues.

Figure 14: Depiction of Three Identified Means of Genomic Interference



This figure depicts the three modes of genomic interference that give rise to aberrant genotyping calls A) When a polymorphism is located under an oligonucleotide, it will alter binding affinity, hence reducing the signal intensity. A spectrum of affinities is possible which makes the spread of genotyping clusters greater. B) When the assays for two SNPs are in close proximity, the oligos will overlap and interfere with each other. C) Homology in the genome to the region of interest will cause a reduction in specificity for the oligos. This can cause incorrect binding and amplification and disrupt the signal for the desired region.

Figure 15: Examples of Genotyping Results Before and After the Incorporation of Novel Design Strategies.



A) Degenerate Base strategy: 1) shows the clustering with standard design. Circled are data points with lower signal intensity due to a SNP underlying the red labeled oligo. 2) shows clustering after inclusion of degenerate bases into oligo design

B) Genotyping results generated from spiking in PCR product in place of genomic DNA for regions of homology. 1) SNP in CYP2C9 from PCR product 2) the detection of the presence of a deleted copy of CYP2D6, three samples circled are positive for the deletion.

C) Separate OPA (read on single array) 1) and 2) show performance of SNPs when assayed in the same pool for two SNPs in close proximity to each other. 3) and 4) show performance when hybridization and ligation occur in separate reactions.

D) Multiple Syntheses. 1) and 2) shows clustering for the same SNP from two different oligo syntheses. 1) all of the signal is coming from the red labeled oligo, 2) all of the signal is coming from the blue labeled oligo. 3) Show clustering from pooled synthesis.

3.5.3 Phase 3

To adapt to the observations made in the second phase of the study and to continue with our ADME development strategy, the final assay design not only integrated our previous design strategies, but also incorporated a double synthesis of each of the OPA oligo pools to compensate for random oligo synthesis failures. Currently, we have used the third version of the assay on three clinical sample sets totaling more than 300 samples. The average marker conversion rate on these samples is 97.6%. The improved SNP assay conversion rates observed for this latest panel design are generating a more complete set of informative ADME markers and thus resulting in a more complete data set that gives improved detection power for the ADME assay.

The 3rd design iteration of the ADME panel fills in many of the gaps in coverage, present on currently available commercial genotyping assays. As much as 90% of the final content of the ADME panel is unique to the panel in comparison to commercially available whole genome products (see Table 3).

3.6 Validation

3.6.1 Genotype Concordance

Forty-two samples from the international HapMap project were run on the final iteration of the ADME panel (7 CEU trios and 7 YRI trios) in order to test for concordance. There are 2953 markers on the ADME panel which are also present in HapMap database (release #2). The individual sample call rates for each SNP were ~96% within the ADME panel data where as the HapMap data is only ~83% complete for the same samples. Thus, the HapMap data has significant gaps in these key ADME genes. After removing missing genotypes, markers that fail hardy-weinberg equilibrium (p-value > 0.05) the concordance between our data and that of the

HapMap is 99%. In 2606 of the 2953 SNPs (88.3%), the concordance is 100%. After the initial analysis, we took a closer look at the discordant SNPs (from the comparison between our ADME panel data and the HapMap data), we observed a significant increase in the number of homozygous allele calls in that HapMap SNPS and have concluded that this is due to a significant allelic dropout (error) in the HapMap data (p-value = 0.02). It appears that other discordant SNPs calls are due to erroneous SNP calls in the HapMap database. This may be due to limitations in the various technology platforms that were used to generate the HapMap data as the majority of discordant SNPs were run on different platforms than the Illumina GoldenGate platform. For example, when we remove the Perlegen data from the overall HapMap data, the concordance goes up to 99.3%.

As a further validation of the ADME panel, an additional 149 samples that had previously been genotyped for 34 of the key functional markers were tested individually using various technologies to definitively determine the accuracy of our multiplexed broad based ADME panel. After analysis, the panel was shown to have a concordance of 99% for 5066 independent genotype calls. These samples had also been genotyped using the Illumina HumanHap300 whole genome array and the concordance of overlapping markers between the two arrays was >99% for ~17,000 genotypes compared.

3.6.2 Cross Technology Comparison

Data from the broad based ADME panel has also been cross validated by comparing it against validated ADME panels developed on two different technological platforms: the Affymetrix DMET Plus Premier Pack[26] and the Illumina VeraCode ADME Core Panel[247]. In total, 85 SNPs are present on all three assays and the concordance between all three assays is

>99.5% in 24 samples screened on all three assays.

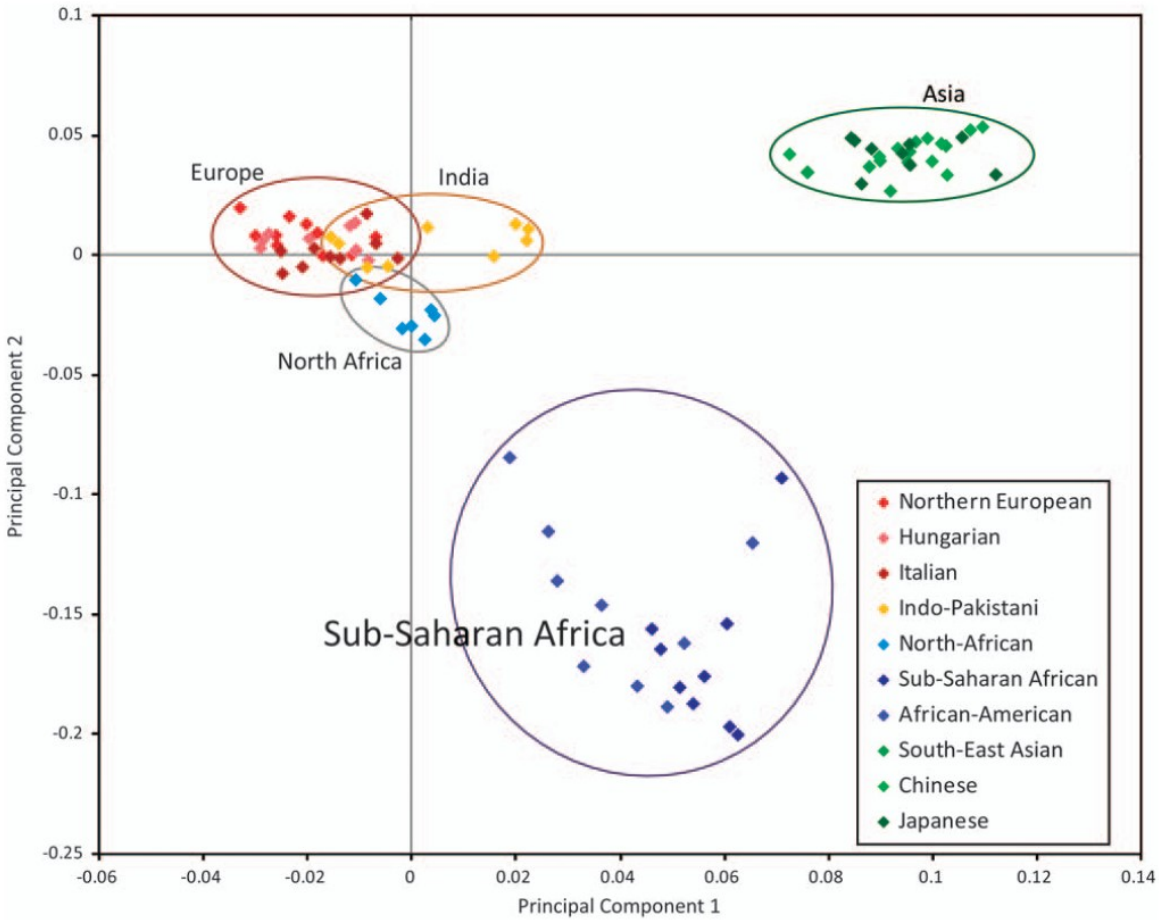
3.7 Discussion

Over three phases of assay development, we have developed a broad based ADME genotyping panel that incorporates multiple strategies that successfully overcome several sources of genomic interference inherent to the GoldenGate assay; underlying polymorphism, SNPs in close proximity and regions of high genomic homology. This improved assay design has permitted the genotyping of SNPs within many ADME regions that were previously very difficult or impossible to genotype. Additionally, SNP genotyping space has been left open within the assays giving researchers the flexibility to spike in additional markers or PCR product in place of genomic DNA. This gives the assay flexibility to be tailored to the needs of a specific study. The consecutive improvements integrated into each iteration of the ADME panel have increased the overall assay conversion rate from 83% using the standard designs and methods to 97% using our modified designs. This increase in marker conversion rate directly impacts SNP coverage and thus improves the panel's power for detection and analysis[266].

After the first phase of ADME panel design, the assay had an overall marker conversion rate of 83%. Even with this lower, non-optimal, number of working assays, *Visscher et al.* demonstrated that the content of the assay was sufficient to be used in principal component analysis to stratify samples by geographic origin (see Figure 16) [267]. Additionally, the first iteration of the broad based ADME assay was also used in an effort to identify genetic causes of adverse drug reactions in children. In an initial cohort of 54 children treated in pediatric oncology units, with replication in a second cohort of 112 children, genetic variants in TPMT (rs12201199, P value = 0.00022, OR = 17.0, 95% CI 2.3-125.9) and COMT (rs9332377, P value

= 0.00018, OR = 5.5, 95% CI 1.9-15.9) were associated with cisplatin-induced hearing loss in children (see table 14) [258]. The results of this study demonstrate that the content and design of the assay were successful in identifying novel observations in pharmacogenomic research studies.

Figure 16: Use of Principal Component Analysis with the ADME panel



A plot of the 1st vs 2nd principal component based on genotype data generated using the ADME panel on a set of worldwide reference samples from the Coriell Institute (Camden, New Jersey). As expected, the pattern resembles the geographic map locations of the typed populations .

Source: Visscher, H. *et al.* 2009

During the second phase of development, we integrated several assay optimization strategies in the new improved design that addressed multiple failure modes present in the first version of the ADME panel assay. The new version of the ADME assay increased conversion rate from 83% to 93.5%. The utility of the assay design and content was further validated in a study of 156 anthracycline-treated patients, with replication in two additional cohorts of 188 and 96 patients for the identification of significant association of a synonymous coding variant rs7853758 (L461L) within the SLC28A3 gene with anthracycline-induced cardiotoxicity (odds ratio, 0.35; $P = 1.8 \times 10^{-5}$ for all cohorts combined) (see table 15)[268]. When combined with additional clinical characteristic, patients could be successfully stratified into low and high risk groups [268]. Once again, the ADME candidate gene approach and our functional plus tagging SNP design proved to be successful at identifying novel genetic biomarkers for this clinical study.

Table 14: Genetic Variants Associated with Cisplatin-Induced Hearing Loss.

			Discovery (n=53)			**Replication** (n=109)			**Combined** (n=162)			
		Geno-	Ototox.	Controls		Ototox.	Controls		Ototox.	Controls		
Gene	**SNP**	**type**	(n= 33)	(n= 20)	**OR (95%CI)**	(n= 73)	(n= 36)	**OR (95%CI)**	(n= 106)	(n= 56)	**OR (95%CI)**	**p-value†**
TPMT	rs12201199	A/A	1	0	2.51 (0.1, 65.00)	2	0	3.09 (0.14, 66.17)	3	0	4.77 (0.24, 94.11)	0.277
		A/T	8	0	14.22 (0.77, 261.62)	14	1	8.60 (1.08, 68.26)	22	1	14.94 (1.96, 114.09)	0.000607
		A/-	9	0	15.90 (0.87, 290.02)	16	1	9.82 (1.25, 77.37)	25	1	16.98 (2.23, 128.99)	**0.000181**
		T/T	24	20	1	57	35	1	81	55	1	
COMT	rs4646316	G/G	20	10	21.48 (1.08, 426.70)	51	15	6.80 (0.58, 80.28)	71	25	19.88 (2.33, 169.70)	**0.000982**
		G/A	13	5	27.00 (1.27, 575.95)	21	19	2.21 (0.19, 26.38)	34	24	9.92 (1.14, 85.95)	0.0215
		G/-	33	15	23.77 (1.24, 457.45)	72	34	4.24 (0.37, 48.34)	105	49	15.00 (1.80, 125.29)	0.00263
		A/A	0	5	1	1	2	1	1	7	1	

Source: *Ross et al.* 2009

Table 15: Risk and Protective Variants for Anthracycline-Induced Cardiotoxicity

				Single marker test		Multi marker model	
SNP rs-ID	Gene	Chr	Position[a]	OR (95% CI)	P-value	OR (95% CI)	P-value
Risk variants							
rs6759892	UGT1A6	2	234,383,669	1.77 (1.20 - 2.61)	0.0038	2.93 (1.74 - 4.94)	0.000022
rs1149222	ABCB4	7	86,718,426	1.87 (1.20 - 2.92)	0.0054	2.31 (1.34 - 4.00)	0.0023
rs4148350	ABCC1	16	16,077,978	3.44 (1.65 - 7.15)	0.0012	3.77 (1.46 - 9.75)	0.0051
rs17583889	HNMT	2	138,579,771	1.91 (1.21 - 3.02)	0.0057	2.21 (1.22 - 4.01)	0.0088

Protective variants							
rs7853758	SLC28A3	9	84,130,480	0.31 (0.16 - 0.60)	0.00010	0.20 (0.09 - 0.45)	0.0000071
rs2020870	FMO2	1	167,886,617	0.14 (0.03 - 0.59)	0.00042	0.09 (0.02 - 0.46)	0.00021
rs2019604	SPG7	16	88,143,266	0.39 (0.20 - 0.76)	0.0021	0.33 (0.15 - 0.73)	0.0026
rs9514091	SLC10A2	13	102,512,255	0.43 (0.23 - 0.78)	0.0033	0.41 (0.21 - 0.82)	0.0074
rs4877847	SLC28A3	9	84,175,971	0.60 (0.41 - 0.89)	0.0092	0.54 (0.33 - 0.87)	0.0097

Both single and multi marker regression models included important clinical covariates and the first two principal components using an additive genetic model. Odds ratios are per copy of the minor allele. Results shown are for the combined Canadian cohort. Only variants included in the final multi-marker model are shown.
OR Odds Ratio; CI Confidence Interval
Source: *Visscher et al*. 2011

The second iteration of the assay was also used to screen two pharmaceutical clinical populations. The first involved acute coronary syndrome in 348 patients taking part in the Rosuva-Atorva ACS trial, CENTAURUS with the aim of identifying genetic variation that can be predictive of the inter-individual variability observed in response to rosuvastatin and atorvastatin. The second involved the use of a novel 5-Lipoxygenase inhibitor in the prevention of atherosclerosis in 187 patients. Interesting results have emerged from each of these studies and the data is presently being finalized for future publication.

The successful identification of novel pharmacogenomic association with the use of our broad based ADME panel reaffirms that our panel design, the ADME gene content and our SNP selection methodology of including both tag and functional variants has been proven to be effective. After having screened more than 1,000 samples in the research and clinical setting, and the validation of genotype calls in more than 200 samples, it can be concluded that the assay we have developed has and will continue to be a valuable tool for use in future pharmacogenomic studies. The content and design of the assay sets it apart from currently available commercial assays that contain only functional markers in a small number of genes or do not have adequate coverage across ADME genes.

Having included two types of variation; known and putative functional markers that can be used in an evidence-based approach and haplotype tagging markers that can be used to discover novel associations, we have created a tool with broad applicability in research and drug development. In the future, we plan to utilize the ADME panel in parallel with commercially available whole genome assays to demonstrate the added utility of having a more complete set of ADME genetic variation when evaluating drug related genotype phenotype correlations.

4 Uncovering Drug Metabolism Genotype Phenotype correlations in Human Liver Samples

4.1 Introduction

There are 2 main types of genotyping experimental designs that predominate in drug metabolism (ADME) pharmacogenomic research: functional/evidence based experiments where definitive decisions must be made based on genotype outcome, and investigative research where candidate gene or genome wide SNPs are studied to identify novel biomarkers of efficacy and/or safety. To support this research, two distinctly different types of commercial genotyping assays have been developed by various technology platform providers. For targeted studies, robust genotyping assays with a small number of very specific functional markers have been developed based on the ADME Cole list of genes (see chapters 1 & 2 above for more detail). Two examples include the Illumina VeraCode ADME Core Panel[247] and the Sequenom iPLEX® ADME PGx panel[246]. These panels can be used to identify known metabolic status phenotypes and stratify patient populations in clinical trials. Currently the only way to perform investigative biomarker identification on a genome wide basis is to use whole genome genotyping arrays. These assays can be used to identify novel genetic correlations with drug response phenotypes in a hypothesis free manner. However, genome wide arrays are often missing many key markers in many of the drug metabolism genes due to areas of genomic interference that we have overcome with the ADME panel (see tables 12 and 13 above).

In order to address these analysis gaps, we developed a broad based ADME genotyping assay that encompassed both known functional markers and tag markers across 181 ADME genes (comprised of 3072 SNPs) which could be applicable to both functional and investigative types of studies mentioned above. This assay has been optimized to a conversion rate close to

100%. Its accuracy and consistency has been demonstrated in over 1000 samples screened to date. Additionally, the assay has been successfully utilized to identify and validate novel drug response genotype/phenotype correlations in multiple studies[258,268]. After development of this panel, the main challenge remaining was to establish what added value, if any, does this ~3000 SNP broad based ADME panel have in relation to the commercial focused core panels and the genome wide assays. Thus, one of the main questions that we wished to answer as part of this chapter was what advantages does this tool have to make discoveries over others? Also, where does this ADME panel fit in relation to other genotyping tools and how can it most optimally be used?

To this end, 150 well characterized liver samples from the Institut für klinische Pharmakologie (IKP) in Stuttgart were screened against our 3000 SNP ADME panel. The livers have been previously tested using several genomic approaches such as whole-genome analysis (318 237 SNPs on the Illumina HumanHap300 Genotyping BeadChip) and candidate SNP genotyping (with a limited number of key SNPs), mRNA gene expression (44 800 mRNA probes with the Illumina Human-WG6v2 Expression BeadChip) and protein expression (using selected antibodies) for selected ADME genes as well as measures of enzymatic activity for a number of ADME enzymes. Additionally, there is also clinical and demographic information available for each sample including disease status at time of surgery and concomitant medications. Liver samples are ideally suited for testing with the ADME panel because this is the organ that is primarily responsible for drug metabolism (see section 1.3.3 above) and therefore, genetic variation in ADME genes that affect gene expression may have a more profound effect in the liver than in other organs and tissue types. Identifying genetic variation associated with gene expression in the liver, will be helpful in elucidating any functional associations that might be

discovered between SNPs and drug response phenotypes. The Stuttgart liver samples represent one of the best collections of characterized liver samples in the world as many previously identified ADME genotype/phenotype observations have already been discovered using these samples[269–271]. In order to determine the added value of our 3000 SNP panel, our strategy was to see to what extent our panel could identify previous genotype/phenotype observations as well as, what additional discoveries, if any, could be made. Uncovering additional genotype/phenotype correlations in the liver samples that have already been extensively tested will show that our ADME panel design strategy and content have added value over currently available commercial assays.

Testing for molecular phenotypes such as gene expression has gained substantial interest recently with the hope of identifying genetic variation with functional consequence that may help explain a greater proportion of the heritability of disease. Several groups have performed large genome-wide studies of gene expression in various tissue and cell types[272–275]. The identification of loci associated with gene expression (expression quantitative trait loci or eQTL) in these studies may help to explain the functionality of association results between SNPs and disease phenotypes. Interestingly, one group found that as many as 30% of eQTLs identified with stringent criteria, failed to replicate in a different cohort of samples[276]. This may partially be explained by variability in experimental protocol and data collection; however it is more likely due to unknown or unmeasured confounding variables[276,277]. This suggests that a significant portion of eQTLs may not be globally informative across studies and cell types. Thus, we decided to perform an eQTL analysis with our ADME panel on these characterized liver cells to see how accurately the data could be reproduced and if any additional observations could be made.

To date, a comprehensive eQTL analysis of ADME gene liver expression with an ADME focused genotyping assay has not yet been performed. Studies have investigated the role of external stimuli such as various drugs on gene expression; others have looked for association between gene expression and drug response phenotypes.[278–280] More recently, the correlation between hepatic expression of certain ADME genes was shown not to be correlated with the expression in blood[281]. *Schadt et al.*[272] and *Innocenti et al.*[276], have each performed a large genome-wide study of gene expression in liver cells using commercially available genome wide SNP arrays and have identified 1229 and 1787 genome wide cis eQTLS respectively. Additionally, *Schröder et al*.[271] used a genome wide SNP array to look for eQTLs in these very samples and identified 979 significant associations, 200 of which were validated based on a comparison to the two previous studies. 89 of the significant cis eQTLs were in ADME genes.

While it is possible to extract ADME variation data, after the fact, from these datasets, as described above, the ADME content on the whole-genome arrays is not very complete (see section 3.3). This is why the application of the ADME panel to the liver samples has the potential to generate a unique dataset of liver ADME eQTLs and will demonstrate the utility our broad based ADME genotyping assay for future studies even when other genotyping efforts have been performed.

4.2 Methods

4.2.1 Samples

DNA derived from 150 liver surgical samples of Caucasian ethnicity (71 male and 79 female) was obtained in collaboration with IKP in Stuttgart[271]. The average age of the samples is

58 ± 14 years. Only non tumor tissue, as determined by a pathologist, was used. Demographic data for the samples, including diagnosis, age and sex, is listed in Table 14.

Table 16: IKP Liver Sample Demographics

Sample	Sex 1=female 2=male	Age	Smoking Status 1=non 2=smoker	Alcohol Use 1=no 2=yes	Ethnicity 1=German 2=Turkish/Greek 3=Unknown	Drugs 1=none 2=P450 Inducer 3=other	Cholestasis*	Diagnosis**
IKP148-016	1	36	1	1	2	1	0	2b
IKP148-018	2	77	2	2	1	3	0	3a
IKP148-019	2	59	1	2	1	3	0	3a
IKP148-021	1	53	na	na	1	3	0	2a
IKP148-022	2	76	1	1	1	3	0	2a
IKP148-025	2	69	1	2	1	3	0	3b
IKP148-028	2	68	1	1	1	3	0	3a
IKP148-029	1	58	2	2	1	2	0	3b
IKP148-036	1	56	1	2	1	2	0	3b
IKP148-038	2	48	1	1	1	3	0	3b
IKP148-039	2	70	1	1	1	3	0	3a
IKP148-040	2	57	1	2	1	3	0	2b
IKP148-041	2	59	2	2	1	3	0	2d
IKP148-042	1	55	2	1	1	3	0	3a
IKP148-043	2	70	1	1	1	1	0	3b

IKP148-044	1	56	1	na	1	3	0	2a
IKP148-047	1	61	2	3	1	3	1	1
IKP148-054	2	57	1	1	1	3	0	3a
IKP148-058	1	54	1	3	1	3	0	3a
IKP148-059	2	57	2	2	1	2	0	2c
IKP148-061	1	71	1	1	1	2	1	3b
IKP148-063	2	73	2	na	1	2	1	2c
IKP148-064	2	63	1	1	1	1	0	3a
IKP148-069	2	85	1	2	1	2	1	3a
IKP148-070	2	71	2	1	1	3	0	2a
IKP148-072	1	59	1	2	1	2	1	2c
IKP148-076	2	66	2	1	1	3	0	3b
IKP148-079	2	7	1	1	1	2	1	2b
IKP148-080	2	74	1	2	1	3	0	3a
IKP148-081	1	74		3	1	3	1	2d
IKP148-082	2	57	1	2	1	1	0	3a
IKP148-085	2	63	1	1	1	3	0	2b
IKP148-087	1	50	1	3	1	3	0	3a
IKP148-096	2	60	1	2	1	2	0	2b

IKP148-098	1	58	2	1	1	1	0	3a
IKP148-102	1	58	1	2	1	3	0	3b
IKP148-103	1	38	1	2	1	1	0	3a
IKP148-104	2	48	na	na	1	3	0	2b
IKP148-106	2	50	1	1	1	1	1	3a
IKP148-118	1	52	2	2	1	1	0	3a
IKP148-120	2	67	1	1	1	3	0	3a
IKP148-121	2	35	2	1	1	1	0	3a
IKP148-122	2	41	1	1	2	1	0	2b
IKP148-123	2	69	1	1	1	2	1	2b
IKP148-124	1	66	2	3	1	3	0	3a
IKP148-126	1	52	1	1	1	3	0	2c
IKP148-127	1	76	2	3	1	2	0	2a
IKP148-129	1	59	1	1	1	1	0	3a
IKP148-132	1	71	2	2	1	2	0	3a
IKP148-133	1	61	1	1	1	1	0	3a
IKP148-134	2	48	1	1	1	3	0	2b
IKP148-135	2	28	1	1	2	3	0	1
IKP148-136	2	77	1	1	1	2	1	2c

IKP148-137	1	50	1	2	1	3	1	2d
IKP148-139	1	74	1	1	1	3	0	2b
IKP148-140	2	39	1	2	1	1	0	2a
IKP148-143	2	72	2	1	1	2	0	3a
IKP148-144	2	54	1	1	1	3	0	2b
IKP148-145	1	52	2	2	1	3	0	3a
IKP148-150	1	59	1	3	1	3	0	3a
IKP148-153	2	44	2	2	2	1	0	3a
IKP148-156	1	53	1	2	1	3	0	3a
IKP148-157	2	54	1	1	1	1	0	3a
IKP148-159	2	56	1	2	1	1	0	2a
IKP148-163	2	69	1	1	1	3	0	3a
IKP148-164	1	43	2	2	3	1	0	2b
IKP148-165	1	47	2	2	1	1	0	3b
IKP148-166	1	70	1	1	1	3	0	3a
IKP148-168	2	77	1	1	1	2	0	3a
IKP148-169	1	67	2	2	1	3	0	3b
IKP148-170	1	15	1	1	1	1	1	1
IKP148-171	1	58	1	1	1	3	0	3a

IKP148-178	1	62	1	2	1	2	1	2b
IKP148-179	2	70	1	1	1	3	1	3b
IKP148-181	2	64	1	2	1	3	0	3a
IKP148-182	2	62	1	2	1	2	0	2a
IKP148-183	1	65	1	1	1	3	0	3a
IKP148-186	2	51	1	1	1	3	0	2a
IKP148-187	2	60	2	2	1	2	0	2a
IKP148-188	2	76	1	1	1	3	0	2a
IKP148-189	1	58	2	1	1	2	0	2a
IKP148-190	2	42	1	2	1	1	0	3b
IKP148-191	2	68	1	2	1	2	0	2b
IKP148-192	1	47	1	1	1	3	0	3a
IKP148-193	2	66	1	1	1	3	0	3b
IKP148-194	2	56	1	1	1	2	0	3b
IKP148-195	1	79	1	3	1	3	0	2a
IKP148-199	1	36	1	2	1	3	0	3a
IKP148-202	2	55	1	2	1	2	1	2c
IKP148-205	1	69	1	3	1	3	0	3a
IKP148-209	2	45	1	2	1	1	0	2b

IKP148-210	2	66	1	1	1	1	0	3a
IKP148-212	1	55	2	2	1	2	1	2c
IKP148-213	1	67	1	2	1	1	0	3a
IKP148-214	1	59	1	2	1	3	0	3a
IKP148-218	2	56	2	3	1	1	0	2b
IKP148-221	2	68	1	1	2	2	0	2b
IKP148-222	2	45	1	1	1	1	0	2a
IKP148-223	2	37	1	1	1	1	0	2b
IKP148-224	1	59	1	1	1	2	0	3a
IKP148-225	1	55	1	1	1	1	0	3a
IKP148-226	2	52	1	1	1	1	1	2b
IKP148-227	2	47	1	1	1	3	0	3b
IKP148-229	1	26	1	1	2	3	1	2b
IKP148-230	2	67	1	1	1	1	0	2b
IKP148-231	2	64	1	1	1	3	0	2d
IKP148-232	2	64	1	1	1	2	0	3a
IKP148-234	1	56	na	na	1	1	1	2c
IKP148-235	2	58	1	1	1	2	1	2c
IKP148-236	2	46	1	1	1	1	0	3a

IKP148-237	1	69	1	na	1	2		2b
IKP148-238	2	75	1	1	1	2	0	2a
IKP148-239	1	54	1	1	1	1	0	3a
IKP148-240	2	42	1	1	1	3	0	3b
IKP148-242	1	51	2	1	1	1	0	2b
IKP148-243	1	69	1	1	1	3	0	2a
IKP148-244	2	74	1	1	1	2	0	3a
IKP148-246	1	45	1	1	1	3	0	3a
IKP148-248	2	48	1	1	1	2	1	2c
IKP148-249	2	72	1	1	1	3	0	3a
IKP148-250	1	57	2	1	1	3	0	2a
IKP148-251	2	50	1	1	1	1	0	3a
IKP148-254	1	72	1	1	1	2	0	3a
IKP148-255	1	60	1	1	1	3	0	3a
IKP148-259	1	70	1	1	1	1	0	3a
IKP148-260	2	74	1	1	1	2		3a
IKP148-261	1	71	1	1	1	3	0	3a
IKP148-264	1	70	1	1	1	1	0	2a
IKP148-266	2	52	1	1	1	3	1	3a

IKP148-268	1	61	1	1	1	2		2c
IKP148-269	1	62	1	1	1	1	0	3a
IKP148-270	2	47	1	1	1	3	0	2b
IKP148-271	2	36	2	1	1	3	0	3b
IKP148-272	2	62	1	1	1	1	0	3a
IKP148-273	1	57	1	1	1	3	0	3a
IKP148-274	1	69	1	1	1	3		2a
IKP148-275	1	62	1	1	1	2	0	3a
IKP148-279	2	40	1	1	1	3	0	2d
IKP148-281	1	57	1	1	1	3	0	2c
IKP148-282	1	32	1	1	1	2	1	2b
IKP148-283	1	65	1	1	1	2	0	3a
IKP148-285	1	43	1	1	1	1	0	3a
IKP148-288	2	72	1	1	1	2	0	2a
IKP148-293	2	80	1	1	1	2	1	2c
IKP148-295	2	75	1	1	1	2	0	3a
IKP148-296	1	72	1	1	1	3	1	2d
IKP148-297	1	52	2	1	1	3	0	3a
IKP148-298	2	32	1	1	1	3	0	2b

IKP148-299	1	56	1	1	1	1	0	3a
IKP148-300	1	63	1	1	1	3	0	3a

*cholestasis as described in *Nies et al*., Hepatology 2009; 50:1227-1240;
**Diagnosis: 1=no tumor 2=primary tumor of liver (2a=HCC, 2b=liver tumor, 2c=klatskin, 2d=gallbladder), 3=metastasis (3a=colorectal, 3b other)

4.2.2 Genotyping Data

DNA from the 150 liver samples, described above, was obtained from IKP Stuttgart and was genotyped using our broad based ADME panel (see Appendix 2 for ADME panel protocol). Due to limitations in the quantity of liver sample DNA, only the pooled OPAs were used to screen the samples, and thus, no spike-in experiments where performed with these samples because this requires substantially more DNA. Using the final iteration of the ADME panel design (outlined above), each sample was screened for 3000 ADME SNPs.

4.2.2.1 Genotyping Quality Control

The genotype clusters were visually inspected using GenomeStudio v2010.1(Illlumina). 77 SNPs were removed from further analysis due to poor clustering. Additionally, 193 SNPs were excluded from the analysis for deviating from HWE (p –value <0.05) and 237 SNPs were removed for having a minor allele frequency < 0.05. In total 2496 SNPs remained for further analysis. The breakdown of theses SNPs is as follows: 41 Core Functional SNPs, 320 Putative Functional / Tag SNPs, and 2135 Tag SNPs.

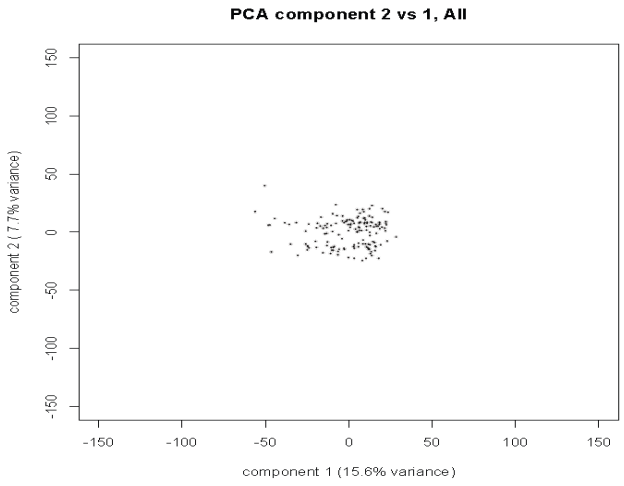
4.2.3 Liver Gene Expression Data

The mRNA expression levels for the liver cells were determined as described in *Schroder et al.* [271] In summary, genome wide mRNA expression was assessed in the liver samples using the Illumina HumanWG-6 V2 array. The array consists of 48 701 probes spanning the genome. The data was preprocessed using Illumina BeadStudio version 3.0 and signal intensities were calculated. The expression intensity data was then quantile normalized and log2 transformed.

4.2.3.1 Gene Expression Quality Control

In order to test for probes with outlier expression data across all samples, principal component analysis (PCA) was performed on the expression data for all 150 samples (see Figure 17). No samples were excluded based on results of the PCA.

Figure 17: Plot of the 1st vs 2nd principal component for expression data from 48701 probes on the 150 samples.



In order to increase the confidence in the association analysis, two additional quality control steps were performed on the expression probes to remove potentially bad data. The first step involved making sure that probes mapped uniquely to their target gene. Probes without a "perfect" mapping score in the ReMOAT database were removed [282]. Secondly, all probes with a potential underlying SNP (dbSNP130) were removed from the data set (see Table 12). After processing the data for these QC steps, 17 971 probes were removed because they did not perfectly map to their target gene and 4 412 probes were removed because they contained potentially underlying SNPs. Thus, after data clean-up, 26 320 probes remained for analysis.

Table 17: Summary of Probe QC

	Total	Non Perfect Probes	Perfect Probes with Underlying SNPs
Number of Probes	48 703	17 971	4 412
Probes Remaining for Analysis	48 703	30 732	**26 320**

4.2.4 Additional Phenotypes

In addition to mRNA expression data several other phenotypes were available for analysis. Additional clinical data is listed in Table 18. No additional clinical information impacted the association results when used as covariates in the analysis.

Table 18: IKP Liver Sample; Additional Clinical Data

Sample	Bilirubin (mg/dl)	alkaline phosphatase (IU/L)	gamma-glutamyl transferase (IU/L)	Aspartate aminotransferase (IU/L)	Alanine aminotransferase (IU/L)	Albumin (g/dl)	CRP (mg/dl)
IKP148-016	0.6	58	19	8	17	4.9	0.6
IKP148-018	0.9	227	na	28	33	na	2.3
IKP148-019	0.4	95	13	11	8	4.8	0.8
IKP148-021	0.4	112	59	16	6	4.7	0.5
IKP148-022	0.3	346	130	19	11	4.2	0.5
IKP148-025	0.9	479	455	34	36	3.8	1.1
IKP148-028	0.4	110	10	6	4	4.1	0.5
IKP148-029	0.3	136	34	17	6	3.8	7.2
IKP148-036	0.6	116	32	11	6	4.9	0.5
IKP148-038	0.5	114	11	12	16	4.9	0.5
IKP148-039	0.6	221	37	17	21	4.8	0.5
IKP148-040	0.6	97	9	14	18	4.6	0.5
IKP148-041	1.3	177	22	21	45	5.2	0.7
IKP148-042	0.65	129	37	7	4	4.5	0.6

IKP148-043	0.4	194	7	9	4	4.7	0.5
IKP148-044	1.9	112	42	31.5	54	5	0.9
IKP148-047	3.3	157	35	7	7	4.1	19.3
IKP148-054	0.6	202	16	10	1	4.5	0.5
IKP148-058	0.5	106	33	11	19	4.9	0.5
IKP148-059	0.9	331	101	28	37	4.3	0.8
IKP148-061	1.6	227	73	118	111	4.4	0.95
IKP148-063	1.2	877	427	29	82	4.1	0.8
IKP148-064	0.3	115	22	12	15	4.2	0.5
IKP148-069	0.5	921	213	44	84	3.7	12.5
IKP148-070	0.4	92	18	11	5	3.5	0.5
IKP148-072	3.2	445	258	24	49	4	4
IKP148-076	0.2	187	38	14	17	3.9	0.4
IKP148-079	8	977	93	69	44	2.7	21.1
IKP148-080	0.6	139	11	24	31	4.6	0.1
IKP148-081	0.7	851	605	62	109	4	10.8
IKP148-082	0.5	230	49	10	8	3.7	6.4
IKP148-085	0.4	202	42	13	20	4.5	1
IKP148-087	0.6	134	53	22	44	4.4	0.3
IKP148-096	0.9	98	6	8	7	4.4	0.3
IKP148-098	1.05	198	33	15	16	4.2	1.05
IKP148-102	0.7	127	27	17	38	4.3	0.4
IKP148-103	0.9	62	10	10	7	4.5	0.1
IKP148-104	0.8	111	26	9	15	4.9	0.2
IKP148-	0.8	775	239	62	79	4.4	1.7

106							
IKP148-118	0.4	201	29	14.5	14	4.6	2.6
IKP148-120	0.3	176	na	11	5	na	2.2
IKP148-121	0.5	127	10	8	6	3.9	1.2
IKP148-122	0.5	89	7	6	4	4	0.1
IKP148-123	1.7	269	80	52	25	2.8	31.1
IKP148-124	0.2	132	15	12	9	4.3	0.1
IKP148-126	0.4	143	177	15	27	4.5	0.1
IKP148-127	0.7	134	39	17	14.5	3.9	1.85
IKP148-129	0.3	123	16	11	12	4.2	1.1
IKP148-132	0.4	175	19	7	6	3.2	1.9
IKP148-133	0.7	141	24	12	11	4.2	1.2
IKP148-134	0.45	72.5	7	10	11	3.8	0.1
IKP148-135	0.8	73	9	19.5	46	4.4	0.1
IKP148-136	6.6	1680	1550	152	206	3.4	5
IKP148-137	41.55	407	140	21.5	30.5	na	4.55
IKP148-139	0.65	100	53	10.5	7.5	4.5	0.1
IKP148-140	0.7	144	18	10	7	4.5	0.4
IKP148-143	0.4	85	11	9	5	4.2	0.9
IKP148-144	0.4	126	74	12	10	4.7	1
IKP148-145	0.6	180	63	7	7	4.1	3.2
IKP148-150	1.4	231	79	16	21	4.5	0.1
IKP148-153	0.5	111	19	12	19	4.3	0.1
IKP148-156	1	230	137	14	15	4.2	1.5
IKP148-157	0.9	122	20	13	20	4.5	0.1

IKP148-159	0.6	121	57	11	16	4.4	0.9
IKP148-163	0.4	171	60	12	11	4.4	0.3
IKP148-164	0.9	92	15	10	9	4.3	0.1
IKP148-165	0.5	88	21	11	14	4.3	0.1
IKP148-166	0.8	93	12	11	8	4.3	0.25
IKP148-168	0.2	168	54	10	10	3.6	6.1
IKP148-169	0.45	271	140	8	8	3.7	2.6
IKP148-170	0.9	242	29	65	104	4.6	3.5
IKP148-171	0.3	407	105	16	28	3.6	4.1
IKP148-178	3.1	376	246	38	77	3.6	4.2
IKP148-179	3.45	1381.5	245	24.5	16	2.4	5.2
IKP148-181	1.3	81	13	8	6	4.4	0.1
IKP148-182	0.5	148	59	16	42	3.6	3.4
IKP148-183	0.4	155	65	10	8	4.6	0.1
IKP148-186	0.3	325	54	21.5	41	4.6	0.4
IKP148-187	0.8	623	155	74	60	3.7	4.8
IKP148-188	1	286	100	26	17	3.4	1.7
IKP148-189	0.4	117	35	10	11	3.2	1.2
IKP148-190	0.4	86	14	9	6	4.7	0.1
IKP148-191	0.3	287	120	17	20	3.4	7.6
IKP148-192	0.9	71	25	12	12	3.9	0.1
IKP148-193	0.55	219	46	13	11.5	4.3	0.5
IKP148-194	0.9	75	9	14	22	4.1	0.1
IKP148-195	0.7	295	72	12	21	4	0.2
IKP148-	0.5	159	67	16	26	4.5	0.1

199							
IKP148-202	1.2	1080	136	19	21	3.2	6.6
IKP148-205	0.5	91	23	18	29	4.4	0.1
IKP148-209	0.75	63	8	7.5	7	4.5	0.1
IKP148-210	0.8	171	20	13	10	4.3	1.3
IKP148-212	2.8	163	16	11	6	4.4	1
IKP148-213	0.3	100	32	6	6	4	2.8
IKP148-214	0.4	206	33	9	9	3.9	2.1
IKP148-218	0.7	585	332	19	29	4.5	4.2
IKP148-221	0.5	197	19	10	10	4.3	1.6
IKP148-222	0.8	208	61	60	55	3.9	0.3
IKP148-223	0.6	89	12	10	5	4.6	0.1
IKP148-224	0.6	196	28	15	18	4.6	0.5
IKP148-225	1.1	103	21	8	3	4	0.1
IKP148-226	1.8	400	168	24	116	3.6	0.2
IKP148-227	0.9	125	10	13	18	4.3	0.1
IKP148-229	0.8	157	136	66	134	4.7	0.1
IKP148-230	0.7	171	20	13	10	4.3	0.1
IKP148-231	0.5	451	40	27	41	3.7	2.3
IKP148-232	0.8	434	120	21.5	21	20.8	13.6
IKP148-234	4.4	649	740	22	19	3.3	10.1
IKP148-235	3.7	467	112	36	51	3.8	0.1
IKP148-236	0.7	127	11	14	11	4.7	0.1
IKP148-237	na	na	na	21	Na	na	na
IKP148-238	1.1	232	154	37	26	3.8	1

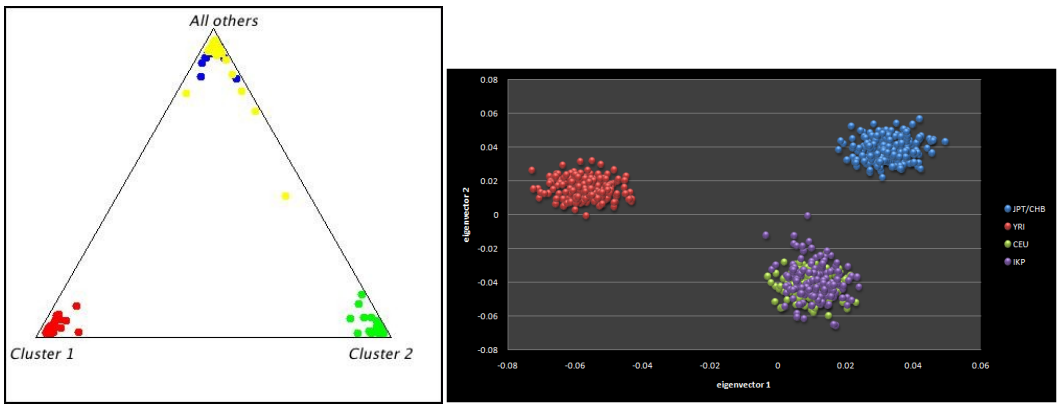
IKP148-239	0.4	89	24	10	10	4.5	0.1
IKP148-240	0.2	111	11	9	6	3.9	0.6
IKP148-242	0.4	124	10	12	14	3.7	0.1
IKP148-243	0.5	141	30	14	15	4.3	0.5
IKP148-244	0.2	92	18	7	5	4.1	1.4
IKP148-246	1	122	na	15	10	na	0.1
IKP148-248	0.4	259	108	75	190	3.9	2.7
IKP148-249	1	177	16	8	2	3.8	0.1
IKP148-250	0.6	124	39	7	5	4.2	0.1
IKP148-251	0.2	104	9	10	8	3.9	0.3
IKP148-254	0.6	146	20	15	10	4.4	0.1
IKP148-255	0.5	201	33	9	12	4.7	0.3
IKP148-259	0.5	94	40	13	16	4.1	0.1
IKP148-260	na	na	na	12	Na	na	0.1
IKP148-261	0.5	106	22	9	9	3.7	0.3
IKP148-264	0.9	91	39	23	43	4.6	0.1
IKP148-266	1.7	na	na	135	184	na	na
IKP148-268	2.4	1043	230	31	Na	3	0.6
IKP148-269	4	122	51	38	54	4.2	0.8
IKP148-270	0.5	173	44	8	6	4.4	0.1
IKP148-271	0.4	122	19	8	9	4.2	0.7
IKP148-272	0.7	129	13	17	24	4.1	0.1
IKP148-273	0.4	108	8	7	6	4.1	0.1
IKP148-274	na	na	na	33	Na	na	na
IKP148-	0.3	202	78	16	23	3.7	4.9

275							
IKP148-279	0.4	589	56	15	20	3.9	1
IKP148-281	0.6	103	7	8	8	4.2	0.1
IKP148-282	2.1	847	191	85	97	3.8	0.9
IKP148-283	1	261	96	35	15	4.4	2.1
IKP148-285	0.4	201	30	12	19	4.3	1.9
IKP148-288	0.6	103	20	10	7	4	0.1
IKP148-293	1.1	1068	661	42	82	3.7	4
IKP148-295	0.9	199	137	12	13	4	0.1
IKP148-296	5	449	51	27	39	3.4	0.1
IKP148-297	0.3	119	15	11	18	4.6	0.1
IKP148-298	0.6	125	64	14	16	4.6	0.1
IKP148-299	0.6	231	114	12	19	4.2	1
IKP148-300	0.9	118	18	12	8	4.9	0.1

4.2.5 Population Stratification

Two independent methods were used to assess if there was any underlying population stratification in the Stuttgart liver samples; the software program Structure and PCA (see Figure 18) were used to assess the population structure in the liver samples using ~600 SNPs in linkage equilibrium (r-squared <0.2) from the ADME panel that were also common to the HapMap project[283,284]. HapMap samples of known ancestry (CEU, YRI and JPT/CHB) were used as reference populations. Both methods identified two individuals (IKP148-016 and IKP148-168) that were phenotypically labeled as CEU but which fell outside the CEU cluster. These two individuals were excluded from future analysis.

Figure 18: Results from STRUCTURE and PCA analysis of Liver Samples



On the left CEU samples are in blue, YRI samples are in red, JPT/CHB samples are in green and IKP samples are in yellow. On the right, CEU samples are in green, YRI samples are in red, JPT/CHB samples are in blue and IKP samples are in purple. Using both methods two individuals fall outside the CEU cluster.

4.2.6 Statistical Analysis

Plink v1.06 was used to test for association between each SNP and each probe (2496 SNPs x 2632 probes = 6 569 472 tests). Linear regression using additive model was performed using age and sex as covariates. Analysis was restricted to 3 data sets:

1. All probes vs all SNPs
2. All SNPs vs probes within +- 100 kb
3. All SNPs vs probe in the same ADME annotated gene

4.2.7 Multiple Testing Correction

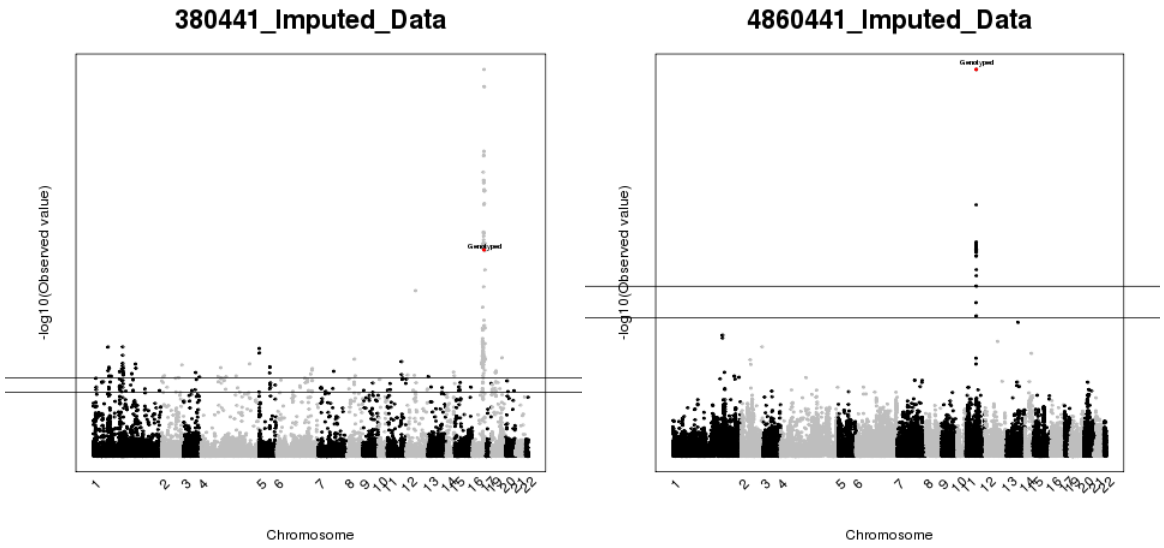
Two methods were used to account for false positives that may arise by chance due to the number of tests. P value significance thresholds were set using a Bonferroni correction that takes into account the number of SNPs (p-value 2.0e-5 for 3000 SNPs) and the number of tests (p-value 7.611e-10 for ~66 million tests) being performed, and the false discovery rate calculation

(FDR) was used (10% FDR p-value 2.158e-07, 5% FDR p-value 6.635e-08, 1% FDR p-value 8.053e-09).

4.2.8 Imputation as Quality Control

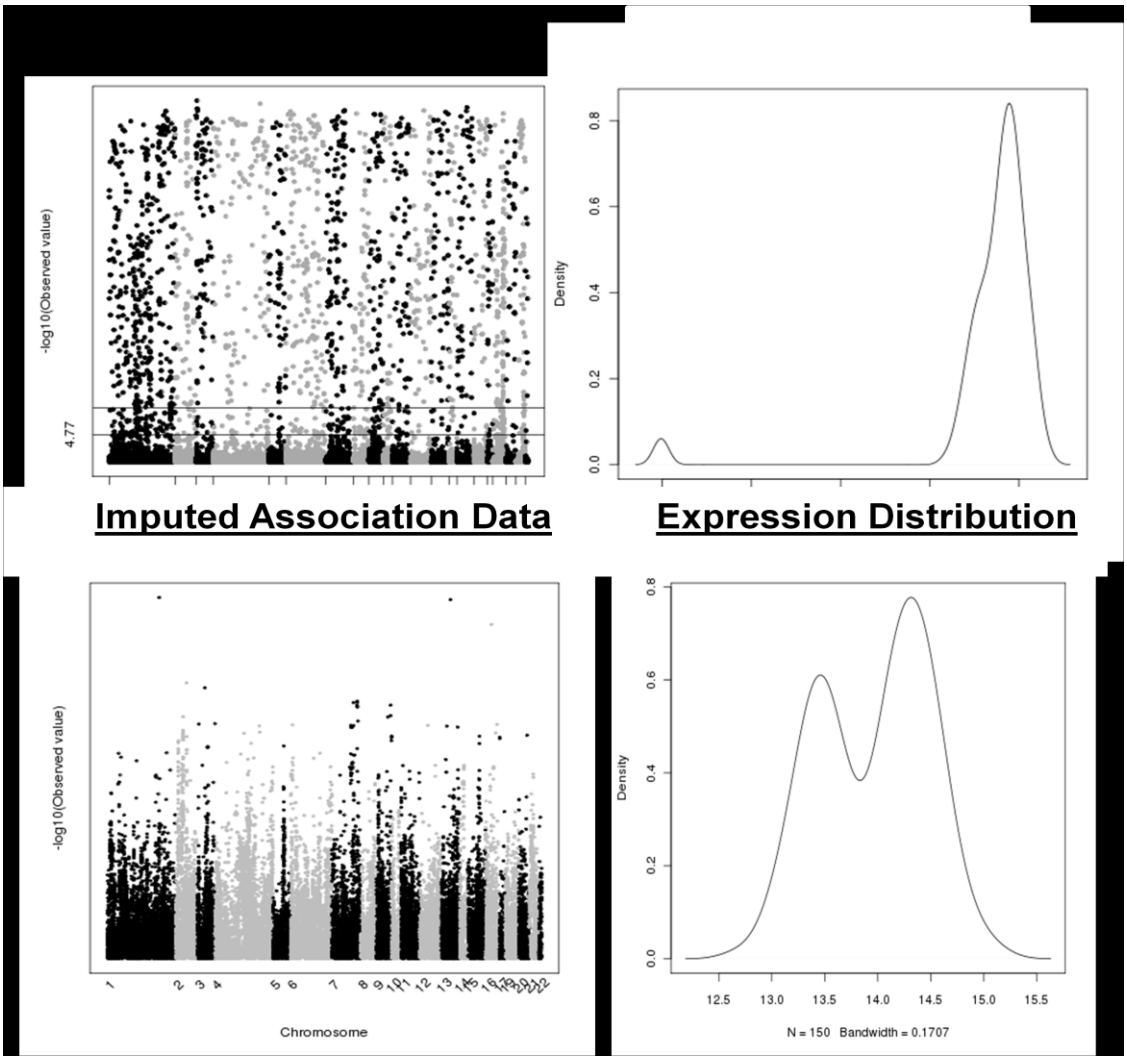
SNPs from HapMap3 and the 1000 genome project (CEU population) were imputed for regions spanning 20 kb upstream and downstream of each gene included on the ADME panel using Impute2[285]. The genotype/phenotype associations were calculated with the imputation data using SNPTEST[122]. The imputation association data was used to prioritize the original association results. When a lone spike was observed in the imputed data the association was determined to be reliable (see Figure 19).Using imputation and association data, the following probes (5700477, 50392 and 4540424) were flagged as being erroneous because of unusual patterns using this method of analysis and were subsequently removed from the final data set (see Figure 20 and Table 19).

Figure 19: Examples of Good Imputation Association Results



Genotyped SNP with smallest p value is indicated in red. For probe 380441 the imputed SNPs have more significant p values, for probe 4860441 a genotyped SNP has the most significant p value. Horizontal lines indicate p values of 2.0e-5 and 7.611e-10

Figure 20: Example of Flagged Imputation Data



The association for probes 50392 and 5770047 were flagged after observing the imputation data. Upon further investigation the expression of these probes was not normally distributed which gave rise to the spurious associations.

Table 19: Flagged Association Data based on Imputation Data

PROBE	GENE	CHR	SNP	P	Previously Identified
5700477	DDT	22	rs575959	3.24E-46	
5700477	DDT	22	rs113413	9.07E-29	***Zeller et al.* (2010)**
50392	SULT2A1	19	rs296381	6.23E-22	
50392	SULT2A1	19	rs188440	3.65E-10	
4540424	EPHX2	8	rs1042064	2.13E-07	

4.3 Results

4.3.1 Cis eQTLs

The data analysis was focused primarily on cis associations as trans associations are difficult to prove; however future analysis will more thoroughly investigate this data. Cis acting associations were defined as SNPs and gene probes being within 100 kb of each other. Using a Bonferroni significance threshold of 7.611 e-10, there were 22 significant cis associations. At a 1% FDR there remained 25 significant cis associations. There are 68 cis associations with a p value smaller that 2.0r-05 (Bonferroni correction for 3000 SNPs). These 68 associations equate to 60 SNPs that correlate with eQTLS for 32 genes (See Table 20). 15 of the cis eQTLs, including the top association, had previously been identified on a different set of samples in one or multiple previously published eQTL studies (highlighted in red Table 20) giving further evidence to the validity of the results.

Table 20: Significant cis eQTLs (p-value <2.0e-05)

SNPs that are lower than the 1%FDR cutoff (8.053e-09) have a green background, 5%FDR cutoff (6.635e-08) have a blue background and 10% FDR cutoff (2.158e-07) have an orange background.

PROBE	GENE	CHR	SNP	P	Previously Identified
6330037	CHURC1	14	rs7143764	2.30E-62	*Veyrieras et al. (2008) Stranger et al. (2007)*
5700477	DDT	22	rs575959	3.24E-46	
6330037	CHURC1	14	rs1886506	2.16E-41	*Veyrieras et al. (2008) Stranger et al. (2007)*
6840497	GSTT1	22	rs113413	5.78E-30	*Zeller et al. (2010)*
380441	ABCC11	16	rs16945988	6.74E-29	
5700477	DDT	22	rs113413	9.07E-29	*Zeller et al. (2010)*
6330037	CHURC1	14	rs8016174	2.05E-25	*Veyrieras et al. (2008) Stranger et al. (2007)*
4860441	SLC22A10	11	rs502642	6.48E-23	
50392	SULT2A1	19	rs296381	6.23E-22	

1340653	SLC22A10	11	rs502642	5.14E-20	
6840497	GSTT1	22	rs575959	1.38E-19	*Stranger et al. (2007)*
3830538	GPX7	1	rs835344	8.49E-19	*Zeller et al. (2010)*
620280	ARNT	1	rs11204735	6.02E-15	*Stranger et al. (2007)*
620280	ARNT	1	rs10888395	2.00E-14	*Schadt et al. (2007)*
6590689	GSTO2	10	rs568526	2.21E-14	
6330037	CHURC1	14	rs2296327	9.29E-14	*Stranger et al. (2007)*
4860441	SLC22A10	11	rs556730	1.96E-13	
1050035	GSTA3	6	rs10948726	3.98E-13	
3830538	GPX7	1	rs946154	1.40E-12	
1340653	SLC22A10	11	rs556730	4.82E-12	
3930189	SLC22A3	6	rs641990	4.72E-11	
6130669	PSMB9	6	rs1383268	1.09E-10	*Zeller et al. (2010)*
50392	SULT2A1	19	rs188440	3.65E-10	
380441	ABCC11	16	rs17743256	4.55E-10	
380441	ABCC11	16	rs8050120	5.56E-09	
7320632	SLC25A27	6	rs953062	3.50E-08	
6130669	PSMB9	6	rs4148882	3.72E-08	*Zeller et al. (2010)*
6590343	PERLD1	17	rs1053651	9.12E-08	
6590689	GSTO2	10	rs966645	9.27E-08	
7320632	SLC25A27	6	rs12192544	1.17E-07	*Schadt et al. (2007)*
5670722	FMO4	1	rs6662297	1.18E-07	
5670722	FMO4	1	rs714839	1.69E-07	
4540424	EPHX2	8	rs1042064	2.13E-07	
6040754	ENTPD5	14	rs7153587	2.60E-07	
4050008	TDRD6	6	rs9472817	3.63E-07	
3370300	CYP3A7	7	rs4646450	5.56E-07	
6060390	CDA	1	rs1689924	7.95E-07	
1050035	GSTA3	6	rs9395826	1.06E-06	
6370497	ULK3	15	rs2470890	1.31E-06	*Zeller et al. (2010)*
6040754	ENTPD5	14	rs1536	1.37E-06	
6130669	PSMB9	6	rs1383266	1.43E-06	
6370382	IQCB1	3	rs2293616	1.47E-06	
1340653	SLC22A10	11	rs4570581	2.13E-06	
1470050	GSTZ1	14	rs7972	2.14E-06	
6550328	PON1	7	rs854552	2.54E-06	
6370382	IQCB1	3	rs1143672	2.89E-06	
6370382	IQCB1	3	rs2257212	3.34E-06	

3830538	GPX7	1	rs835337	3.72E-06	*Zeller et al. (2010)*
6130669	PSMB9	6	rs6912492	4.41E-06	
1470050	GSTZ1	14	rs2287398	4.45E-06	
3830538	GPX7	1	rs7529595	4.98E-06	
4050008	TDRD6	6	rs953062	5.58E-06	
5670722	FMO4	1	rs2294482	5.76E-06	
6940133	UGT1A3	2	rs6725478	5.97E-06	
4860441	SLC22A10	11	rs4570581	6.58E-06	
6370382	IQCB1	3	rs866926	7.19E-06	
6370497	ULK3	15	rs762551	9.32E-06	
6940133	UGT1A3	2	rs4294999	9.63E-06	
1260470	LONP2	16	rs16945988	9.71E-06	
4180050	FMO1	1	rs714839	1.12E-05	
3780717	UGT2A1	4	rs1432329	1.12E-05	
3930189	SLC22A3	6	rs569919	1.23E-05	
5700477	DDT	22	rs2858908	1.34E-05	
7320632	SLC25A27	6	rs12215229	1.36E-05	
5810598	GNAI3	1	rs542338	1.67E-05	*Zeller et al. (2010)*
2710575	GPX2	14	rs7143764	1.69E-05	
7210017	CYP21A2	6	rs433061	1.79E-05	
3420632	DOPEY2	21	rs2835286	1.92E-05	

4.3.2 Trans Associations

A trans eQTL was defined as any association where the SNP and probe were on different chromosomes. There are 23 significant trans eQTLs using a 10% FDR significance threshold (see Table 21). None of these trans eQTLs had been previously identified and none were in ADME genes.

Table 21: Significant trans eQTLs (p-value <= 2.11E-07)

GENE	CHR	SNP	P
KCNG1	20	rs575959	4.19E-18
KCNG1	20	rs113413	5.08E-12
TSN	2	rs6068810	9.22E-10
PGCP	8	rs6068810	1.08E-09

MLANA	9	rs6068810	2.23E-09
TYRP1	9	rs6068810	3.96E-09
SFRS12	5	rs6068810	5.99E-09
AKT1	14	rs17578180	1.85E-08
LOC347376	X	rs6068810	5.60E-08
PSMA7	20	rs2239566	8.03E-08
RIMS2	8	rs6068810	9.43E-08
RPLP1	15	rs6068810	9.69E-08
FAM98A	2	rs952499	9.75E-08
SFRS11	1	rs12562630	9.81E-08
HTN3	4	rs6068810	1.01E-07
WDSOF1	8	rs2239566	1.05E-07
CARS	11	rs12543000	1.24E-07
TFAP2A	6	rs543613	1.25E-07
GDI2	10	rs2817227	1.33E-07
RPS28	19	rs12553329	1.36E-07
SUMO2	17	rs512077	1.43E-07
ATG16L1	2	rs11635541	1.57E-07
RNF121	11	rs2027055	2.11E-07

4.3.3 ADME Gene Analysis

As we are primarily interested in ADME gene associations, the analysis was further refined to examine only association between SNPs on the ADME panel and probes annotated to be in the same ADME gene. Some SNPs were annotated for an entire loci rather than a single gene due to short inter-genic distances or strong LD (eg UGT1A, and CYP3A). At significance thresholds of 1.32 E-04 (1% FDR) and 9.37E-04 (5% FDR) there are 57 and 79 significant eQTLs respectively, when looking only at ADME genes and SNPs (see Table 22). This corresponds to eQTLS for 24 ADME genes/loci. Of these ADME eQTLs, only four have previously been reported in studies within different sets of samples and only three were previously identified in the same 150 liver samples using a whole genome genotyping array[276].

Table 22: Significant ADME Gene Associations

Previously identified associations from any study are indicated in red. Gene associations previously identified in the IKP150 liver samples are bolded and underlined. 1%FDR: 1.32 E-04 (have a green background) and 5%FDR: 9.37E-04 (have a blue background).

SNP	SNP Gene/Locus	Probe	Probe Gene	P-value
rs16945988	ABCC11/12	380441	ABCC11	6.74E-29
rs502642	SLC22A9/10	4860441	SLC22A10	6.48E-23
rs502642	SLC22A9/10	1340653	SLC22A10	5.14E-20
rs835344	GPX7	3830538	GPX7	8.49E-19
rs11204735	ARNT	620280	ARNT	6.016E-15
rs10888395	ARNT	620280	ARNT	2.003E-14
rs568526	GSTO1/O2	6590689	GSTO2	2.21E-14
rs556730	SLC22A9/10	4860441	SLC22A10	1.955E-13
rs10948726	GSTA1/2/3/4/5	1050035	GSTA3	3.979E-13
rs946154	GPX7	3830538	GPX7	1.399E-12
rs2144698	GSTA1/2/3/4/5	1050035	GSTA3	2.306E-12
rs556730	SLC22A9/10	1340653	SLC22A10	4.817E-12
rs2608632	GSTA1/2/3/4/5	1050035	GSTA3	2.306E-11
rs641990	SLC22A3	3930189	SLC22A3	4.718E-11
rs17743256	ABCC11/12	380441	ABCC11	4.554E-10
rs4715326	**GSTA1/2/3/4/5**	**1050035**	**GSTA3**	**7.654E-10**
rs2608624	GSTA1/2/3/4/5	1050035	GSTA3	9.125E-10
rs8050120	ABCC11/12	380441	ABCC11	5.558E-09
rs966645	GSTO1/O2	6590689	GSTO2	9.272E-08
rs6662297	FMO	5670722	FMO4	1.183E-07
rs714839	**FMO**	**5670722**	**FMO4**	**1.694E-07**
rs1042064	EPHX2	4540424	EPHX2	2.129E-07
rs2180314	GSTA1/2/3/4/5	1050035	GSTA3	4.597E-07
rs4646450	CYP3A	3370300	CYP3A7	5.556E-07
rs1689924	CDA	6060390	CDA	7.946E-07
rs9395826	GSTA1/2/3/4/5	1050035	GSTA3	1.06E-06
rs2741023	UGT1A	6940133	UGT1A1	1.09E-06
rs4570581	SLC22A10	1340653	SLC22A10	2.13E-06
rs7972	GSTZ1	1470050	GSTZ1	2.14E-06
rs2741019	UGT1A	6940133	UGT1A1	2.27E-06
rs854552	PON1	6550328	PON1	2.54E-06
rs835337	GPX7	3830538	GPX7	3.72E-06

rs2287398	GSTZ1	1470050	GSTZ1	4.45E-06
rs7529595	GPX7	3830538	GPX7	4.98E-06
rs2294482	FMO	5670722	FMO4	5.76E-06
rs6725478	UGT1A	6940133	UGT1A1	5.97E-06
rs4570581	SLC22A10	4860441	SLC22A10	6.58E-06
rs4294999	UGT1A	6940133	UGT1A1	9.63E-06
rs714839	**FMO**	**4180050**	**FMO1**	**1.12E-05**
rs1432329	UGT2A1/2B4	3780717	UGT2A1	1.12E-05
rs569919	SLC22A3	3930189	SLC22A3	1.23E-05
rs7143764	GPX2	2710575	GPX2	1.69E-05
rs433061	CYP21A2	7210017	CYP21A2	1.79E-05
rs718016	FMO	5670722	FMO4	2.79E-05
rs3820170	FMO	5670722	FMO4	2.99E-05
rs7877	FMO	5670722	FMO4	3.03E-05
rs4124874	UGT1A1	6940133	UGT1A1	3.26E-05
rs3820170	FMO	4180050	FMO1	4.00E-05
rs13269963	EPHX2	4540424	EPHX2	5.97E-05
rs757158	PON1	6550328	PON1	8.37E-05
rs1319811	UGT2A1/2B4	3780717	UGT2A1	9.74E-05
rs10916827	CDA	6060390	CDA	9.98E-05
rs1569343	UGT2A1/2B4	3780717	UGT2A1	1.08E-04
rs2292334	SLC22A3	3930189	SLC22A3	1.32E-04
rs43047	PON2/3	6450753	PON2	0.0001885
rs2231142	ABCG2	2320711	ABCG2	0.0002369
rs16945874	ABCC11/12	380441	ABCC11	0.0002532
rs43037	PON2/3	6450753	PON2	0.0002767
rs854573	PON1	6550328	PON1	0.000277
rs1886506	GPX2	2710575	GPX2	0.0002772
rs2760139	ALDH5A1	3780524	ALDH5A1	0.0003145
rs2180312	GSTA1/2/3/4/5	1050035	GSTA3	0.0003346
rs2272365	PON1	6550328	PON1	0.0003595
rs17147507	UGT2A1/2B4	3780717	UGT2A1	0.0003925
rs7527068	GPX7	3830538	GPX7	0.000429
rs17863787	UGT1A	6940133	UGT1A1	0.0004464
rs2835286	CBR3	1470132	CBR3	0.0004709
rs6662297	FMO	4180050	FMO1	0.0005119
rs818194	CDA	6060390	CDA	0.0005143
rs2076317	ALDH5A1	3780524	ALDH5A1	0.0005243
rs1823803	UGT1A	6940133	UGT1A1	0.0006196
rs4787456	SULT1A1/2	5270477	SULT1A1	0.0006907

rs2239566	CBR3	1470132	CBR3	0.0008067
rs3091344	CYB5R3	2490678	CYB5R3	0.0008437
rs10499314	SLC22A3	3930189	SLC22A3	0.0008658
rs2288740	UGT2A1	3780717	UGT2A1	0.0009367

When examining eQTL data from the *Schadt et al. paper,* there are 27 cis eQTLs that appear in ADME genes that overlap with genes present on our 3000 SNP ADME panel. Of the 27 genes identified in the *Schadt et al.* data, we identified at least one significant cis eQTL in 8 of these genes in this study: CBR3, GSTO2, PON2, SULT2A1, UGT2A1, ARNT, GSTA3, and FMO1. For comparison, the *Schadt et al.* data was generated using almost 3 times the number of human liver samples (427) as our study of 150 while also using whole genome SNP arrays with more than 780 000 SNPs. Of the remaining 19 genes in the *Schadt et al.* data that did not have cis eQTLs in our data, the average p value 0.06, trending toward significance. Moreover, our study identified an additional 17 cis eQTLs in key ADME genes that are unique to our liver data set, and for which no pervious observations have been made: ABCC11, FMO4, UGT1A1, ABCG2, GPX2, ALDH5A1, GPX7, CDA, GSTZ1, CYB5R3, PON1, CYP3A7, SLC22A10, EPHX2, SLC22A3, CYP21A2, and SULT1A1. In the *Schadt et al.* data, there are three SNPs that are present on our ADME panel that are significantly associated with cis eQTLs that we did not see in our data because probes in these genes were removed as part of our stringent QC process.

While the *Schadt et al.* data was generated on a different set of human liver samples, the *Schröder et al.* eQTL data was generate on the same 150 human liver samples using the Illumina 330 whole genome array. Comparison of both data sets identified ADME cis eQTLS, in the same six ADME genes: ARNT, FMO4, GPX7, GSTO2, SLC22A10 and UGT1A1[271]; however there are an additional 13 ADME genes with cis eQTLs that are unique to our data set: ABCC11,

ABCG2, GPX2, ALDH5A1, CDA, GSTZ1, CYB5R3, PON1, CYP3A7, EPHX2, SLC22A3, CYP21A2, and SULT1A1. Therefore, in a straight comparison study using the same liver samples, our ADME panel had the same ability to detect an equal number of eQTLs (5) as the commercial whole genome arrays while having additional content that increased its sensitivity to discover an additional 13 eQTLs that were not detected using conventional tools.

In addition to mRNA expression, twenty-two additional phenotypes were tested for association with ADME genotype (see Appendix 5). The additional phenotypes include twelve protein expression phenotypes measured by Western blot analysis (CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, P450, POR, UGT1A1, and UGT1A3) and ten enzymatic activities measured using probe drugs (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and POR). The protein level of three genes (UGT1A3, CYP3A5 and UGT1A1) had 23 significant associations with ADME SNPs. For some of these associations, the same SNP was also associated with mRNA expression (See Table 23).

Table 23: Additional Phenotype Associations

Phenotype	SNP	SNP Gene	P-value	eQTL	P-value
UGT1A3	rs4294999	UGT1A	3.73E-19	UGT1A1	0.000009634
UGT1A3	rs6725478	UGT1A	1.08E-18	UGT1A1	0.000005967
UGT1A3	rs4124874	UGT1A1	6.71E-17	UGT1A1	0.00003261
UGT1A3	rs2741023	UGT1A	4.01E-15	UGT1A1	0.000001092
UGT1A3	rs2741019	UGT1A	3.66E-12	UGT1A1	0.000002269
UGT1A3	rs17863787	UGT1A	3.03E-11	UGT1A1	0.0004464
UGT1A3	rs2602378	UGT1A	1.90E-09		
UGT1A3	rs28946889	UGT1A	1.23E-08		
CYP3A5	rs2242480	CYP3A	1.65E-08		
CYP3A5	rs2687079	CYP3A	4.54E-08		

UGT1A3	rs1823803	UGT1A	5.52E-08	UGT1A1	0.0006196
CYP3A5	rs4646450	CYP3A	1.67E-07	CYP3A7	5.556E-07
UGT1A3	rs6431558	UGT1A	3.53E-07		
UGT1A3	rs4148328	UGT1A	5.25E-07		
UGT1A1	rs17863787	UGT1A	5.32E-07	UGT1A1	0.0004464
CYP3A5	rs6945984	CYP3A	6.30E-07		
UGT1A3	rs4281899	UGT1A	7.52E-07		
UGT1A1	rs4294999	UGT1A	1.39E-06	UGT1A1	0.000009634
UGT1A1	rs4124874	UGT1A1	2.51E-06	UGT1A1	0.00003261
UGT1A1	rs6725478	UGT1A	6.21E-06	UGT1A1	0.000005967
UGT1A1	rs2741019	UGT1A	8.86E-06	UGT1A1	0.000002269
CYP3A5	rs472660	CYP3A	8.99E-06		
UGT1A1	rs2602378	UGT1A	1.49E-05		

No significant associations were observed between our ADME SNP genotypes and any other protein expression or enzymatic activity phenotype when treated as continuous or dichotomous trait.

4.4 Discussion

We have completed a thorough investigation of ADME gene eQTLs in samples derived from Human liver tissue. The use of very stringent quality control measures, such as strict HWE and the removal of gene expression probes without perfect mapping scores, may be too conservative and may have resulted in the inadvertent removal of true positive results, however, with such stringent quality metrics we are confident in these observations. Interestingly, eQTLs were observed for three SNPs in the *Schadt et al.* data that are present on the ADME panel, however were not observed in our analysis because the probes in these genes did not pass our QC criteria. If these three probes are kept in the analysis, we also observe the same three significant results. While these may be true associations, it has been demonstrated that the

reasons for probe removal during our QC process, i.e. underlying SNPs and non perfect mapping to their target gene, do cause inaccurate gene expression data[286–289].

The number of significant eQTLs in ADME genes observed in this study, 57 and 79, respectively for significance thresholds of 1.32 E-04 (1% FDR) and 9.37E-04 (5% FDR) is similar in number to studies using whole genome approaches, 65 and 89 for *Schadt et al.* and *Schroder et al.* There are two reasons to explain these findings. First, the testing of only 3000 SNPs on the ADME panel does not suffer the same multiple testing penalty, and therefore has a lower p-value threshold, than experienced when using a whole genome genotyping approach. Secondly, as mentioned above (see Table 12), much of the content of the ADME panel is not included on commercial whole genome arrays. Of the 70 significantly associated ADME SNPs, 30% and 20% of the them, are not accounted for on the whole genome arrays used in the *Schroder et al.* and *Schadt et al.* studies respectively even when accounting for proxy SNPs.

The design and content of the ADME panel has been validated by the fact that we replicated the findings of several other groups by identifying many of the same eQTLs in liver samples and other tissues using a much smaller but targeted set of ADME SNP markers[226,273,275]. These results also validate the replicated eQTLs. More importantly, our ADME panel shows increased sensitivity to identify novel pharmacogenomic biomarkers as it was able to identify an additional 13 novel ADME eQTLs using the same liver samples which had been previously investigated using whole genome genotyping approaches. While these results must be considered tentative until they can be validated in an additional cohort of liver samples; this clearly demonstrates the added value of this assay when compared to commercial whole genome arrays.

This list of ADME eQTLs produced by this analysis has identified a number of genotype/phenotype correlations that needs to be further investigated to determine what impact any of these SNPs has on gene function and altered gene expression. For example, a SNP in a gene encoding a drug metabolism enzyme may be significantly associated with an adverse response to a drug. It may turn out that this SNP is an eQTL for the same drug metabolism gene and carriers of a certain allele have reduced expression. It may then be inferred that the reason for the association with the adverse event is that these people have reduced expression of the gene and therefore reduced metabolism. The generation of the ADME eQLT list will serve a valuable tool for future pharmacogenomic studies.

In addition to ADME eQTLs, associations were identified between ADME protein expression and ADME genotype. While these correlations require future validation, it is interesting that many of the associated SNPs are also ADME eQTLs for the same gene. These results show a potential link between mRNA expression and protein expression via genetic variation. Additionally, one of the SNPs associated with CYP3A5 protein expression, rs6945984, has also been associated with olanzapine clearance, thus implicating a possible mechanism for the association[290].

Finally, many CYP enzymes are known to be highly polymorphic with regard to gene and protein expression and yet none were detected in this analysis. For CYP2D6, CYP2C9 and 2C19, this is due to the fact that the 3000 SNP panel does not include any markers for these genes. The SNPs in these genes require additional PCR enrichment of the targets which are spiked into the reaction (see above). We did not have enough DNA from the liver samples to add the PCR spike in genes. For other genes, such as CYP2A6 and CYP2B6, the mechanism of

expression differences, may involve complicated mechanisms such alternative splicing that may not be well detected using micro arrays[291].

4.5 Conclusion

The result of the application of the broad based ADME panel to identify novel genotype/phenotype correlation, described above, has demonstrated the usefulness of our ADME panel design. Not only were we able to identify previously reported cis eQTLs but we were able to show the added sensitivity and added value of our panel over the other commercially available genome-wide SNP arrays, by identifying eQTLs in ADME gene that had not previously been reported using the very same samples. This is most likely due to the poor coverage of ADME gene on whole genome arrays or to the difficulty in generating good quality genotyping data due to area of genomic interference (discussed above) in areas surrounding many ADME genes.

These results strongly suggest that our ADME panel would have broad applicability to most pharmacogenomic clinical trials and would have greater sensitivity to detect novel genotype/phenotype observations over current commercially available tools. Any study investigating the role of genetic variation on drug response could benefit from the use of our assay in their study.

5 Optimus Primer: A PCR enrichment primer design program for next-generation sequencing of human exonic regions.

Over the course of the ADME panel development, several bioinformatic tools were developed to assist with assay and primer design. In this section, the further development of one of these tools into a utility for next-generation sequencing enrichment experiments is described. Sequencing experiments are becoming more prevalent however, costs for the whole genome sequencing of a large number of samples is still prohibitive. This is why many groups are enriching for certain regions of the genome prior to sequencing experiments.

To facilitate with primer design during the ADME panel development, an automated primer design pipeline was developed. This pipeline was modified to be able to automate the design of exon specific primers for PCR enrichment in sequencing experiments. It was our intention during the ADME panel development, to make bioinformatic tools that could be manipulated with very little effort so that they could have applicability in several areas of genetic research.

As the cost associated with sequencing experiments continues to drop, it may one day be cost effective to do large scale sequencing experiments on thousands of samples rather than genotyping. In this case, the content developed for the ADME genotyping assay could be translated into an ADME sequencing panel and a tool like the one described in this section would help with this process.

Optimus Primer: A PCR enrichment primer design program for next-generation sequencing of human exonic regions.

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5.1 Abstract

5.1.1 Background

Polymerase chain reaction (PCR) remains a simple, flexible, and inexpensive method for enriching genomic regions of interest for next-generation sequencing. In order to utilize PCR in this context, a major challenge facing researchers is how to generate a very large number of functional PCR primers that will successfully generate useable amplicons. For instance, in an exon-only re-sequencing project targeting 100 genes, each with 10 exons, 1,000 pairs of primers are required. In fact, the reality is often more complex as each gene might have several isoforms and large exons need to be divided to maintain the desired amplicon size. With only a list of gene names, our program Optimus Primer (OP) automatically takes into account all these variables, and can generate primers with no need to provide genome coordinates. More importantly however, OP, unlike other primer design programs, uniquely utilizes Primer3 in an iterative manner that allows the user to progressively design up to four iterations of primer designs. Through a single interface, the user can specify up to four different design parameters with

different stringencies, thus increasing the probability that a functional PCR primer pair will be designed for all regions of interest in a single pass of the pipeline.

5.1.2 Findings

To demonstrate the effectiveness of the program, we designed PCR primers against 77 genes located in loci associated with ulcerative colitis as part of a candidate gene re-sequencing experiment. We achieved an experimental success rate of 93% or 472 out of 508 amplicons spanning the exonic regions of the 77 genes. Moreover, by automatically passing amplicons that failed primer design through three additional iterations of design parameters, we achieved an additional 170 successful primer pairs or 34% more in a single pass of OP than by conventional methods.

5.1.3 Conclusion

With only a gene list and PCR parameters, a user can produce hundreds of PCR primer designs for regions of interest with a high probability of success in a very short amount of time. Optimus Primer is an essential tool for researchers who want to pursue PCR-based enrichment strategies for next-generation re-sequencing applications. The program can be accessed via website at http://op.pgx.ca.

5.2 Findings

The development of next-generation sequencing (NGS) technologies has dramatically increased the size and scale of sequencing experiments. It is now possible to produce several gigabases of DNA sequence in a short period of time[292]. To date, the cost of whole human genome sequencing remains prohibitive. Focusing NGS experiments to specific genomic regions is an alternative, cost-effective approach to whole genome sequencing but it requires the enrichment

of the targeted regions before library construction. Several hybridization-based methods – each with its own strengths and weaknesses – have been developed[293]. While these DNA enrichment methods continue to be developed and improved, a simple and inexpensive alternative is PCR. PCR is a robust, well-understood, very accessible and flexible strategy for DNA enrichment. It also allows for the very specific amplification of targeted regions without the high background found in hybridization-based methods. Hundreds or even thousands of PCR amplicons that span selected genomic regions of interest can be pooled together and used as input material for the sequencing reaction. PCR has been traditionally used for classic Sanger chemistry-based sequencing of few genes. However, the throughput of next-generation DNA re-sequencing is such that new tools need to be developed to facilitate the implementation of PCR as enrichment strategy for these new sequencing methods.

Enrichment of exonic regions is of particular interest as the functionality of variations within these regions can be more easily inferred than variations in non-coding DNA. Surveying genetic variation by NGS for all exons in candidate genes, such as those identified in genome wide association studies (GWAS), may contribute to the identification of the causal genes and variants, and therefore the underlying biology of the disease.

In order to utilize PCR in the context of NGS, where hundreds of genes are resequencd, a major challenge facing researchers is how to generate a very large number of functional PCR primers that will successfully generate useable amplicons. For instance, in order to target the re-sequencing of 100 genes, each with 10 exons, this requires 1,000 pairs of PCR primers. In fact, the reality is often more complex as each gene might have several isoforms and the enrichment strategy must accommodate large exons that require overlapping PCR products to maintain the desired amplicon size. Thus, to address this significant bioinformatic need in setting up robust NGS enrichment pipelines, we have developed the Optimus Primer (OP) website (http://op.pgx.ca) where users can import a gene list with no need to provide genome coordinates to generate a complete list of PCR primer pairs (see Figure 21 and Table 24 for example).

Table 24: Example PCR Primer Pairs Designed After Submitting TCF7L2

Region Name	Forward Primer	Reverse Primer
TCF7L2_chr10_exon1-3_1	CTGTTTATTTATGCACACGTCACTG	gTACTCACCTTCTTCCAAACTTTCC
TCF7L2_chr10_exon1-3_2	GATTCTTTTTCTCCCCCTTCTC	AGCCAGCAATCTCCACTAGAAAG
TCF7L2_chr10_exon4_1	TCCCTGTATGCTTATTGAATAGGTG	CCCAGAAAAGAAGTCATGGAATAAC
TCF7L2_chr10_exon5_1	GGCTAGTTGTTCTGCATTTACTTTC	AGCTCCAAAATAAGGAGGCAGTTAG
TCF7L2_chr10_exon6_1	AATAGGAGAGTTCTGATTTGTGCTG	GACTTATTCCACATTTTGTCTCTCC
TCF7L2_chr10_exon7_1	CATCTTAGAAAATCCAGGTGAGAGG	AACAACTGGGATAAAAAGGGGATG
TCF7L2_chr10_exon8_1	ATGAGATGAAACCTACCAACAACTC	TGTTGTTCAGAGTACAGATCACTGG
TCF7L2_chr10_exon9_1	CTTACTGTGCAGAGAGAACTTTTCC	ATTAGCGACTAAAACATACTGCTGC
TCF7L2_chr10_exon10-12_1	ACGATTTACACAGCTTTCTGTCTTC	CTATGTCATTCTGTCATTTGCTCAC
TCF7L2_chr10_exon10-12_2	CTGAGTGCACGTTGAAAGAAAG	AGAGAGGACTAGGCAGATCCTGTAG
TCF7L2_chr10_exon13-14_1	CAGACACTCTTCTCACATCTGTTTC	GGAGGTTTATTACTGAATTCCTTCC
TCF7L2_chr10_exon15-16_1	CTCGCTTCTCTCTTGAACTCATTC	CTTCTCCATGTGTCTCGACTCTAAG
TCF7L2_chr10_exon17_1	TATTCACAGATAACTCTCTCCCCTG	TCTATTAAGTGTTGAGTAGCGTCCC
TCF7L2_chr10_exon17_2	ACATCTGGTTTTTAAACCGTAAGGG	GAGCATAAAACGGAACAGTAACATC
TCF7L2_chr10_exon17_3	CATATTACATACGAGTAGGCAGCAG	CGATACTGTGGTCACCTTAAGAAAC

After having only submitted the gene name (TCF7L2) Optimus Primer was able to successfully design PCR primers to amplify all exons (see figure 1 for amplicons position). Note that exon in close proximity to each other were merged into single amplicons (exons 1-3 for example) and that large exons were divided into several amplicons to maintain consistent amplicons size (exon 17 for example).

Figure 21: Example of Exon Coverage of Optimus Primer Designed Amplicons

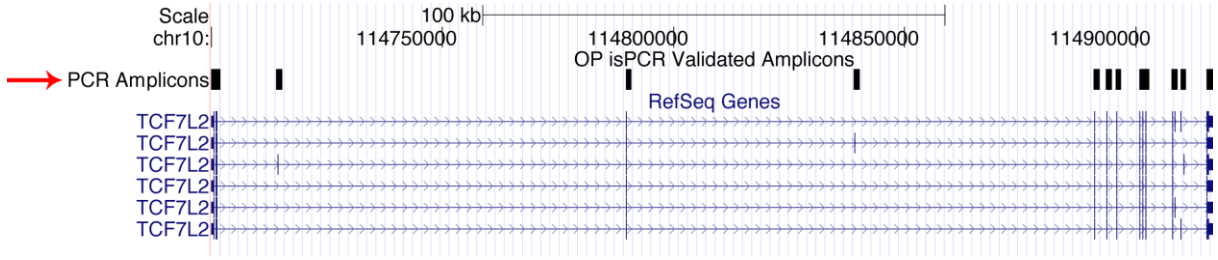

Image from the UCSC genome browser (genome.ucsc.edu) showing Optimus Primer design results for the gene TCF7L2. The black boxes (highlighted by the red arrow) indicate the positions of all isPCR validated amplicons designed by OP. Note that only the gene name, TCF7L2, was submitted into the pipeline yet amplicons were designed spanning the exons (indicated by blue boxed) for all 6 known isoforms of the gene.

Other groups have developed pipelines to facilitate PCR primer design[294–296]. These programs often cannot accommodate primer design from the gene names alone and require significant background research to obtain sequence information. Furthermore, most of these programs are only capable of processing a limited list of genes at a time and lastly, all of these programs do not possess the ability to handle multiple design criteria to design multiple iterations of the same amplicon in a single run, thus maximizing the likelihood that a successful primer pair will be designed. Therefore these algorithms are not suitable for the large number of PCR primers required for NGS enrichment. In contrast, OP is able to handle many genes simultaneously and requires only the gene name as input. The user of the program has the ability to set the design criteria for up to four iterations of primer designing parameters that allows the user to progressively modify the design iterations to make them less stringent in order to design primers for all submitted genes or regions. This makes this program especially useful for the rapid design of the large number of primers needed in targeted resequencing experiments.

5.3 Implementation

Optimus Primer (OP) is a web-based automated pipeline that requires the user to submit only a gene list, or list of regions of interest, and primer design parameters. The pipeline consists of four steps. First, all exons for all known isoforms for each gene submitted are identified using the RefSeq database[297]. This step is skipped if regions of interest are submitted. A list of all unique exons for each gene is then generated with exons/regions that are in close proximity to each other (<25 base pairs for example) merged into a single element in the list. Second, the pipeline extracts the desired genomic sequences from the current build of the human genome (currently hg18/ NCBI36), plus additional flanking sequence at a length defined by the user to facilitate the design of the PCR primers. OP will prioritize the design of the primers to these flanking regions to ensure complete coverage of the specific exonic regions. The user has the option of including or excluding sequence that has been masked with RepeatMasker[298]. Additionally, polymorphisms from the current build of dbSNP (currently build 130) can be masked to ensure that primers are not designed to locations with underlying SNPs[221]. Primer3 has been integrated into the pipeline to design PCR primers using user defined parameters[117]. Exons/regions that are larger than the specified amplicon size will be automatically split into smaller amplicons, with a minimum 25 bp overlap to ensure that every base can be amplified and sequenced. Exons for which no PCR primer design is possible using the initial parameters are passed on to a second iteration of Primer3 with modified design criteria defined by the user.

Currently, the pipeline allows the user to define up to four iterations of Primer3 design criteria in a single pass to attempt to design PCR primers for all amplicons with up to 5 primer pairs for each amplicon. The final step of the pipeline is to run all designed PCR primers through the UCSC Genome Browser in-silico PCR (isPCR) utility as a validation step for the primer pairs

selected[299]. The isPCR utility allows the user to check the human genome for the presence of unique primer pairs, ensures that they are designed correctly on opposite strands, that they are the correct distance apart and generates a report of the theoretical amplicons produced by the primer pair. OP then uses this data to generate a report for all primer designs as well as the percent coverage for each exon/region for each gene for all isPCR validated primer pairs. Primers designed with OP can then be used to amplify genes of interest as the enrichment step prior to library construction for NGS experiments. In particular, because PCR is flexible and easily implementable, OP will be ideal to target for NGS genes that are difficult to enrich using solid- or liquid-based capture reagents and for genes that are very polymorphic. Additionally, for genes whose annotation is dynamic from one build of the human genome to the other, PCR can be easily adapted whereas probes-based capture reagents will need to be re-synthesized.

5.4 Results

As part of a high-throughput DNA re-sequencing project to identify genetic risk factors for ulcerative colitis, we targeted 77 genes for NGS. PCR amplification was selected as enrichment strategy and we opted for pooled sequencing. This corresponds to 867 unique exons and a total of 237 kb of sequence. OP divided the exons into 993 amplicons (average size of 586 bp). After a single pass of OP, primer pairs were designed for 861 (87%) of amplicons using our stringent design parameters (see Table 25).

Table 25: Primer Design Parameters Used in the Four Passes of Primer3.

	First Pass	Second Pass	Third Pass	Fourth Pass
Optimum Size (BP)	25	25	25	25
Minimum Size (BP)	20	20	18	20
Maximum Size (BP)	30	30	33	30
OptimumTM (^{o}C)	60	60	60	60

Minimum TM (^oC)	55	55	50	55
Maximum TM (^oC)	65	65	70	65
Optimum GC%	50	50	50	50
Minimum GC%	40	40	35	40
Maximum GC%	60	60	65	60
GC CLAMP	Yes	No	No	Yes
NUM RETURN	1	1	1	1
PRODUCT SIZE RANGE (BP)	400-600	400-600	400-600	400-600
Repeat Masking	Yes	Yes	Yes	No
SNP Masking	Yes	Yes	Yes	Yes

PCR primers could not be designed for all desired amplicons for reasons such as low complexity DNA and underlying variation. However with additional effort, it is possible to modify (loosen) Primer3 parameters to allow for the design of PCR primers in these regions. 714 of the OP designed primer designs (861) passed our isPCR evaluation and validation process (representing 72.5% of desired exons). In the lab, we tested 508 of the isPCR validated primer pairs using 7 different genomic DNA samples. We demonstrated successful PCR products for 472 amplicons (92.9% success rate) using agarose gel electrophoresis and/or PicoGreen quantification. Of the 508 primer pairs tested, 338, 18, 80 and 72 were designed in the first, second, third and fourth iterations of Primer3 respectively. By including three additional design iterations with different design parameters with different stringencies, it resulted in an additional 170 successful primer pairs or an increase of 34% more designs in a single pass of OP than by conventional methods. The success rates were 93%, 84%, 99% and 97% for the four iterations. Therefore, we feel that our approach of using multiple design iterations with progressively looser design parameters is a valid approach to successful primer design. In our specific experiment, amplicons were pooled and libraries were constructed according to Illumina's recommendations. We re-sequenced the targeted exons using a single-end 36-base pair protocol on our Genome Analyzer II. We

generated 4.5 gigabases of DNA sequences, 89% of which was on target. This allowed us to generate 40X coverage and identify over 700 coding DNA sequence variants. Specific details from genetic risk factors for UC will be presented elsewhere.

5.5 Conclusion

PCR is currently the cheapest, simplest, most flexible approach for sample enrichment prior to NGS experiments. It also has some distinctive advantages over the less specific enrichment methodologies currently used for targeted next generation resequencing. In order to capitalize on PCR-based methodologies, hundreds if not thousands of PCR primers need to be designed. To address this gap in bioinformatic tools, we have developed Optimus Primer (OP), a web-based automated PCR design pipeline that facilitates the simultaneous design of PCR primers for the enrichment of exonic regions in multiple genes. This tool can be useful not only for the enrichment of exonic regions for NGS experiments, but it also has much more general applicability to other experiments that require the rapid design of PCR primers for multiple regions of interest such as genotyping, Sanger sequencing and real time PCR. With only a gene list and PCR parameters, a user can design hundreds of PCR primers in a very short timeframe.

5.6 Authors Contributions

AMKB, KSL and PG wrote all software, created the website and drafted the manuscript. MB performed the PCR and validation experiments. GL conceived the project and helped draft manuscript. J-CT, MSP and JDR participated with the design and coordination of the project, and helped draft the manuscript. All authors read and approved the final manuscript.

5.7 Acknowledgments

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6 Summary & Perspectives

The work presented within this thesis describes the development and utilization of the types of tools needed to continue to expand the field of pharmacogenomics. To date pharmacogenomic research has predominantly focused on improving the use of drugs that already have regulatory approval; however the data that is being generated such as that presented, is gradually changing the drug development process and a future of drugs developed in parallel with a companion genetic test is not too far off.

6.1 Future of Pharmacogenomics

The extent to which genomic personalized medicine becomes part of routine medical practice depends on how the ethical, legal, economic and medical issues that surround the various stakeholders are accounted for. These stakeholders include: the patients, the doctors, the diagnostic test providers, the pharmaceutical companies and the payers, either governments or insurance companies.

Patients want a drug that will work for them without adverse reactions. They also want their genetic information kept secure and do not want it to affect them in a negative way such as being refused insurance or employment[300]. Doctors require genetic testing that is accessible and easy to interpret and understand. Additionally, alternative treatments are necessary to make pharmacogenetic testing viable as it will be difficult for physicians to not treat a patient with a given drug if a patient's genetic profile indicates that there is a probability that they will not respond. What does one do if there is no alternative treatment available? The education of physicians also needs to be updated to include the advances being made in pharmacogenomics. In a recent survey of Canadian physicians, the majority of respondents felt that genetic testing will have a positive effect on the treatment of patients; however half of theses respondents

thought that there is currently not enough evidence to support the incorporation of genetic testing into routine care[301]. Finally, informatics infrastructure needs to be established to assist with the interpretation, transfer and storage of genetic data in the clinic. The companies that produce the genetic tests need to be reimbursed for the time and money spent developing and getting regulatory approval tests that are accurate and interpretable [302]. Pharmaceutical companies do not want to have their market share reduced if they are to continue to spend billions of dollars on the development of new drugs. Healthcare payers do not want to pay for unnecessary medical testing and want the results of genomics medicine to be conclusive and must help to lower costs in the long run. The potential to do so is there. Finally, regulatory bodies, such as Health Canada and the FDA need to adapt to the rush of new technologies and biomarker testing in order to take advantage of all facets of personalized medicine. Specifically, regulatory agencies will need to adapt their current processes to incorporate new drugs that have been developed using genomic based clinical trial designs which have companion diagnostic tests that must accompany the launch of the drug[303].

It is clear that there is support from all parties mentioned above for personalized medicine to become a reality, and as mentioned earlier, there are several examples of its utilization currently. As we continue to grow the body of knowledge surrounding genetic variation and its effects on drug response, gradually, the use of genetic information in the prescribing of drugs will become more widespread and accessible.

7 Conclusion

The goal of the work presented in this thesis was broken down into four areas. The first was to establish consensus within academia and the pharmaceutical industry on a set of ADME genes and markers that should become the basis for pharmacogenomic studies involving any medication. The second was to develop and validate a genotyping assay that could be used to screen this set of ADME variation. Third was to demonstrate the utility of this assay in research and clinical studies to validate its effectiveness. Finally, to show that there is added benefit of the assay even in studies where standard genomic analysis methods are used.

The first goal was accomplished through collaboration with the PharmaADME working group, where a Core and Extended list of ADME genes and variation was established to identify a consensus list of key functional ADME variants that can be used to make clinical decisions in drug development and the clinical setting. This list has become the basis for several commercially available assays and it can be argued that no pharmacogenomic study is complete unless these genes and markers are investigated. The PharmaADME.org website has become one of the main resources for this information. The genotyping assay itself was developed in an iterative manner that led to a better understanding of the types of genomic interference that has hindered the analysis of ADME variation in the past. The resulting assay, that utilizes novel design strategies to overcome genomic interference has been optimized and validated. Bioinformatic tools created during this process were also further developed and made publically available to assist with the design of any genetic study. As next generation sequencing is becoming more integrated into research and clinical studies, the same difficulties assaying ADME genes that were seen over the course of this work will be evident and the insight gained in this study will assist with future assay designs. The effectiveness of our design strategies were also

demonstrated in the successful utilization of the assay in the screening of over 1000 samples which has led to novel associations with ADME gene variation and adverse drug reactions in children; specifically, the role of TPMT and COMT with cisplatin-induced hearing loss in children [258] and SLC28A3 with anthracycline-induced cardiotoxicity[268]. Furthermore, the added utility of the assay compared to currently available commercial assays was demonstrated by screening 150 liver samples that have been extensively investigated using many different "omic" approaches. In these samples we were able to validate previously reported cis eQTLs in 13 ADME genes and uncover an additional 13 ADME genes with novel cis eQTLs, doubling the number previously identified using standard methods on these samples.

The success of a pharmacogenomic study depends heavily on the quality of the clinical information that is obtained and the analytical tools which are used to carry out the study. It can be very difficult and time consuming to recruit a quality set of patients for a pharmacogenomic phenotype, therefore it is important to have high quality tools available to test these patients with in order to maximize the return on your efforts. We have gone to great lengths to develop, optimize and validate a targeted ADME genotyping tool, with broad applicability to both pharmacogenomic research studies and clinical trials, in order to provide the pharmacogenomic community with the type of high quality tool needed to conduct their research. It will be necessary now to continue to find ways to utilize these tools in well phenotyped clinical populations. In the end, this will lead to a better understanding of how genetic variation affects drug response, and will change the way drugs are developed and used.

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Appendix 1: Table of FDA Approved Drugs with Pharmacogenomic Information in Their Label

Drug	Therapeutic Area	Biomarker	Label Sections
Abacavir	Antivirals	HLA-B*5701	Boxed Warning, Contradindications, Warnings and Precautions, Patient Counseling Information
Aripiprazole	Psychiatry	CYP2D6	Clinical Pharmacology, Dosage and Administration
Arsenic Trioxide	Oncology	PML/RAR?	Boxed Warning, Clinical Pharmacology, Indications and Usage, Warnings
Atomoxetine	Psychiatry	CYP2D6	Dosage and Administration, Warnings and Precautions, Drug Interactions, Clinical Pharmacology
Atorvastatin	Metabolic and Endocrinology	LDL receptor	Indications and Usage, Dosage and Administration, Warnings and Precautions, Clinical Pharmacology, Clinical Studies
Azathioprine	Rheumatology	TPMT	Dosage and Administration, Warnings and Precautions, Drug Interactions, Adverse Reactions, Clinical Pharmacology
Boceprevir	Antivirals	IL28B	Clinical Pharmacology
Brentuximab Vedotin0	Oncology	CD30	Indications and Usage, Description, Clinical Pharmacology
Busulfan	Oncology	Ph Chromosome	Clinical Studies
Capecitabine	Oncology	DPD	Contraindications, Precautions, Patient Information
Carbamazepine	Neurology	HLA-B*1502	Boxed Warning, Warnings and Precautions
Carisoprodol	Musculoskeletal	CYP2C9	Clinical Pharmacology, Special Populations
Carvedilol	Cardiovascular	CYP2D6	Drug Interactions, Clinical Pharmacology
Celecoxib	Analgesics	CYP2C9	Dosage and Administration, Drug Interactions, Use in Specific Populations, Clinical Pharmacology

Cetuximab	Oncology	EGFR	Indications and Usage, Warnings and Precautions, Description, Clinical Pharmacology, Clinical Studies
Cetuximab	Oncology	KRAS	Indications and Usage, Clinical Pharmacology, Clinical Studies
Cevimeline	Dermatology and Dental	CYP2D6	Drug Interactions
Chlordiazepoxide and Amitriptyline0	Psychiatry	CYP2D6	Precautions
Chloroquine	Antiinfectives	G6PD	Precautions
Citalopram	Psychiatry	CYP2C19	Drug Interactions, Warnings
Citalopram	Psychiatry	CYP2D6	Drug Interactions
Clobazam	Neurology	CYP2C19	Clinical Pharmacology, Dosage and Administration, Use in Specific Populations
Clomiphene	Reproductive and Urologic	Rh genotype	Precautions
Clomipramine	Psychiatry	CYP2D6	Drug Interactions
Clopidogrel	Cardiovascular	CYP2C19	Boxed Warning, Dosage and Administration, Warnings and Precautions, Drug Interactions, Clinical Pharmacology
Clozapine	Psychiatry	CYP2D6	Drug Interactions, Clinical Pharmacology
Codeine	Analgesics	CYP2D6	Warnings and Precautions, Use in Specific Populations, Clinical Pharmacology
Crizotinib	Oncology	ALK	Indications and Usage, Warnings and Precautions, Adverse Reactions, Clinical Pharmacology, Clinical Studies
Dapsone	Dermatology and Dental	G6PD	Indications and Usage, Precautions, Adverse Reactions, Patient Counseling Information
Dasatinib	Oncology	Ph Chromosome	Indications and Usage, Clinical Studies, Patient Counseling Information
Desipramine	Psychiatry	CYP2D6	Drug Interactions
Desloratadine and Pseudoephedrine	Allergy	CYP2D6	Clinical Pharmacology

Dexlansoprazole	Gastroenterology	CYP2C19	Clinical Pharmacology, Drug Interactions
Dexlansoprazole	Gastroenterology	CYP1A2	Clinical Pharmacology
Dextromethorphan and Quinidine	Neurology	CYP2D6	Clinical Pharmacology, Warnings and Precautions
Diazepam	Psychiatry	CYP2C19	Drug Interactions, Clinical Pharmacology
Doxepin	Psychiatry	CYP2D6	Precautions
Drospirenone and Ethinyl Estradiol	Reproductive	CYP2C19	Precautions, Drug Interactions
Erlotinib	Oncology	EGFR	Clinical Pharmacology
Esomeprazole	Gastroenterology	CYP2C19	Drug Interactions, Clinical Pharmacology
Fluorouracil	Dermatology and Dental	DPD	Contraindications, Warnings
Fluoxetine	Psychiatry	CYP2D6	Warnings, Precautions, Clinical Pharmacology
Fluoxetine and Olanzapine	Psychiatry	CYP2D6	Drug Interactions, Clinical Pharmacology
Flurbiprofen	Rheumatology	CYP2C9	Clinical Pharmacology, Special Populations
Fluvoxamine	Psychiatry	CYP2C9	Drug Interactions
Fluvoxamine	Psychiatry	CYP2C19	Drug Interactions
Fluvoxamine	Psychiatry	CYP2D6	Drug Interactions
Fulvestrant	Oncology	ER receptor	Indications and Usage, Patient Counseling Information
Galantamine	Neurology	CYP2D6	Special Populations
Gefitinib	Oncology	CYP2D6	Drug Interactions
Gefitinib	Oncology	EGFR	Clinical Pharmacology
Iloperidone	Psychiatry	CYP2D6	Clinical Pharmacology, Dosage and Administration, Drug Interactions, Specific Populations, Warnings and Precautions
Imatinib	Oncology	C-Kit	Indications and Usage, Dosage and Administration Clinical Pharmacology, Clinical Studies
Imatinib	Oncology	Ph Chromosome	Indications and Usage, Dosage and Administration, Clinical Pharmacology, Clinical Studies
Imatinib	Oncology	PDGFR	Indications and Usage, Dosage and Administration, Clincal

			Studies
Imatinib	Oncology	FIP1L1-PDGFR?	Indications and Usage, Dosage and Administration, Clinical Studies
Imipramine	Psychiatry	CYP2D6	Drug Interactions
Indacaterol	Pulmonary	UGT1A1	Clinical Pharmacology
Irinotecan	Oncology	UGT1A1	Dosage and Administration, Warnings, Clinical Pharmacology
Isosorbide and Hydralazine	Cardiovascular	NAT1; NAT2	Clinical Pharmacology
Lapatinib	Oncology	Her2/neu	Indications and Usage, Clinical Pharmacology, Patient Counseling Information
Lenalidomide	Hematology	Chromosome 5q	Boxed Warning, Indications and Usage, Clinical Studies, Patient Counseling
Maraviroc	Antivirals	CCR5	Indications and Usage, Warnings and Precautions, Clinical Pharmacology, Clinical Studies, Patient Counseling Information
Mercaptopurine	Oncology	TPMT	Dosage and Administration, Contraindications, Precautions, Adverse Reactions, Clinical Pharmacology
Metoprolol	Cardiovascular	CYP2D6	Precautions, Clinical Pharmacology
Mivacurium	Musculoskeletal	Plasma Cholinesterase gene (BCHE) Pseudocholinestrase deficiency	Clinical Pharmacology, Special Populations, Precautions, Warnings
Modafinil	Psychiatry	CYP2C19	Drug Interactions
Modafinil	Psychiatry	CYP2D6	Drug Interactions
Nefazodone	Psychiatry	CYP2D6	Drug Interactions
Nelfinavir	Antivirals	CYP2C19	Drug Interactions, Clinical Pharmacology
Nilotinib	Oncology	Ph Chromosome	Indications and Usage, Patient Counseling Information
Nilotinib	Oncology	UGT1A1	Warnings and Precautions, Clinical Pharmacology

Nortriptyline	Psychiatry	CYP2D6	Drug Interactions
Omeprazole	Gastroenterology	CYP2C19	Dosage and Administration, Warnings and Precautions, Drug Interactions
Panitumumab	Oncology	EGFR	Indications and Usage, Warnings and Precautions, Clinical Pharmacology, Clinical Studies
Panitumumab	Oncology	KRAS	Indications and Usage, Clinical Pharmacology, Clinical Studies
Pantoprazole	Gastroenterology	CYP2C19	Clinical Pharmacology, Drug Interactions, Special Populations
Paroxetine	Psychiatry	CYP2D6	Clinical Pharmacology, Drug Interactions
Peginterferon alfa-b	Antivirals	IL28B	Clinical Pharmacology
Perphenazine	Psychiatry	CYP2D6	Clinical Pharmacology, Drug Interactions
Phenytoin	Neurology	HLA-B*1502	Warnings
Pimozide	Psychiatry	CYP2D6	Warnings, Precautions, Contraindications, Dosage and Administration
Prasugrel	Cardiovascular	CYP2C19	Use in Specific Populations, Clinical Pharmacology, Clinical Studies
Pravastatin	Cardiovascular	Genotype ApoE2/E2 in Fredrickson Type III; Other Hyperlipidemias	Clinical Studies, Use in Specific Populations
Propafenone	Cardiovascular	CYP2D6	Clinical Pharmacology
Propranolol	Cardiovascular	CYP2D6	Precautions, Drug Interactions, Clinical Pharmacology
Protriptyline	Psychiatry	CYP2D6	Precautions
Quinidine	Antiarrhythmics	CYP2D6	Precautions
Rabeprazole	Gastroenterology	CYP2C19	Drug Interactions, Clinical Pharmacology
Rasburicase	Oncology	G6PD	Boxed Warning, Contraindications
Rifampin, Isoniazid, and Pyrazinamide	Antiinfectives	NAT1; NAT2	Adverse Reactions, Clinical Pharmacology
Risperidone	Psychiatry	CYP2D6	Drug Interactions, Clinical Pharmacology
Sodium Phenylacetate and	Gastroenterology	UCD (NAGS; CPS; ASS; OTC; ASL;	Indications and Usage, Description, Clinical

Sodium Benzoate		ARG)	Pharmacology
Sodium Phenylbutyrate	Gastroenterology	UCD (NAGS; CPS; ASS; OTC; ASL; ARG)	Indications and Usage, Dosage and Administration, Nutritional Management
Tamoxifen	Oncology	ER receptor	Indications and Usage, Precautions, Medication Guide
Telaprevir	Antivirals	IL28B	Clinical Pharmacology
Terbinafine	Antifungals	CYP2D6	Drug Interactions
Tetrabenazine	Neurology	CYP2D6	Dosage and Administration, Warnings, Clinical Pharmacology
Thioguanine	Oncology	TPMT	Dosage and Administration, Precautions, Warnings
Thioridazine	Psychiatry	CYP2D6	Precautions, Warnings, Contraindications
Ticagrelor	Cardiovascular	CYP2C19	Clinical Studies
Timolol	Opththalmology	CYP2D6	Clinical Pharmacology
Tiotropium	Pulmonary	CYP2D6	Clinical Pharmacology
Tolterodine	Reproductive and Urologic	CYP2D6	Clinical Pharmacology, Drug Interactions, Warnings and Precautions
Tositumomab	Oncology	CD20 antigen	Indications and Usage, Clinical Pharmacology
Tramadol and Acetaminophen	Analgesics	CYP2D6	Clinical Pharmacology
Trastuzumab	Oncology	Her2/neu	Indications and Usage, Precautions, Clinical Pharmacology
Tretinoin	Dermatology and Dental	PML/RAR?	Boxed Warning, Dosage and Administration, Precautions
Trimipramine	Psychiatry	CYP2D6	Drug Interactions
Valproic Acid	Psychiatry	UCD (NAGS; CPS; ASS; OTC; ASL; ARG)	Contraindications, Precautions, Adverse Reactions
Vemurafenib	Oncology	BRAF	Indications and Usage, Warning and Precautions, Clinical Pharmacology, Clinical Studies, Patient Counseling Information
Venlafaxine	Psychiatry	CYP2D6	Drug Interactions
Voriconazole	Antifungals	CYP2C19	Clinical Pharmacology, Drug Interactions

Warfarin	Hematology	CYP2C9	Dosage and Administration, Precautions, Clinical Pharmacology
Warfarin	Hematology	VKORC1	Dosage and Administration, Precautions, Clinical Pharmacology

Source : (http://www.fda.gov/drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.html

Appendix 2: ADME Panel Modified Golden Gate Method

The following is the Golden Gate method used for the custom ADME panel. Sections that are underligned are deviations from the standard Illumina method.

Required Equipment
- Heat block (Illumina, with 96-well insert for 0,2ml plate)
- Plate shaker (Illumina)
- Raised bar magnetic plate (Illumina, *Dynal catalog # MPC®-96S*)
- Plate Centrifuge
- Hybridization Oven (Illumina)
- 8-channel or 12-channel Multi-pipette (P20 and P200)
- Heat sealer
- Single channel (P200) Pipette
- Single channel (P1000) Pipette
- Autoloader (Illumina)
- iScan (Illumina)
- Tube vortexer
- 96-well thermal cycler with heated lid
- BeadChip Hyb Cartridge (Illumina)
- Grip n' Strip Tool (Illumina)
- BeadChip Coverslips (Illumina)
- Filter plate adaptor (*Millipore catalog #MACF 09604)*
- OmniTray (*Nunc, catalog # 242811)*

Consumables

- 96-well 0.2 mL skirted microplates (AB gene, cat no: AB-0800 or equivalent)
- 96-well cap mat (ABgene, cat no: AB-0566)
- Foil Heating Seal (ABgene, cat no AB-0559)
- 50 mL reservoir basin
- Centrifuge tubes (50 mL)
- Microseal™ "F" film
- Microplate heat seals
- Adhesive films (Phenix Research, cat no. 100SEALPLT)
- Filter plates *MultiScreen Filter Plates, 0.45 µM, clear, Styrene, Millipore (catalog #MAHV-N45 10/50)*
- 96-well V-bottom plates *Costar* Brand 96-Well Plates, Polypropylene*
- *Vbottom plate (VWR International catalog # 29444-102)*
- 96-well storage mats (*VWR International, catalog # 29445-122)*
- Canned air
- Hyb Chambers (X2, Illumina)
- Hyb Chambers gaskets (X2, Illumina)
- Hyb Chambers inserts (X8, Illumina)
- Multi-Sample BeadChip Alignment Fixture
- BeadChips (12x1, Illumina)
- Illumina pre-printed Barcodes labels for chips, Plates, Reagents (etc)

Chemicals

- DNA plate (min 50 ng/μL)
- GS#-MS1 (1 tubes, Illumina)
- 100% 2-propanol (2 mL)
- GS#-PS1 (1 mL, Illumina)
- GS#-RS1 (1.2 mL, Illumina)
- GS#-OPA (1 tubes, Illumina)
- GS#-OB1 (1 tubes, Illumina)
- GS#-AM1 (11 mL, Illumina)
- GS#-UB1 (6ml and 11 mL, Illumina)
- GS#-MEL (1 tubes, Illumina)
- Illumina-recommended DNA Polymerase (64 μL)
- UDG *Uracil DNA Glycosylase (OPTIONAL)* (50 μL, Illumina)
- GS#-IP1 (1 tubes, Illumina)
- GS#-MPB (1 tubes, Illumina)
- 0.1N NaOH (16 mL)
- GS#-UB2 (16 mL, Illumina)
- GS#-MH1 (3.84 mL, Illumina)
- GS#-CHB (2 tubes, 1tube per 48 samples, Illumina)
- GS#-XC4 (1 bottle, Illumina)

If two oligo pools (either standard or PCR product) are being processed simultaneously that are to be merged and read on a singly array it is during step 6. Inoc PCR, that this will occur. The plates for the two pools should be processed at the same time during this step. In order not to confuse which plates are to be merged, it is useful to label the plates that are to be merged with the same number except for an additional 1 and 2 for each step of the protocol. For example: ASE-9999-1 and ASE-9999-2.

1. Make SUD (Pre-PCR Area)

This process activates sufficient DNA of each individual sample to be used *ONCE* in the GoldenGate genotyping assay.

1.1 Preheat the heat block(s) to 95°C (may take up to 45 minutes).

1.2 Thaw **GS#-MS1** to room temperature.

1.3 Vortex to fully mix tube contents and pour into a sterile trough.

1.4 Apply a **GS#-SUD** barcode label to a 96-well 0.2 mL plate.

1.5 Add 5 μL **GS#-MS1** reagent to each well of **GS#-SUD** plate.

If genomic DNA is being activated follow step 1.6a. If PCR product is being activated follow step 1.6b.

Genomic DNA

1.6a Transfer 5 µL normalized DNA sample to each well of the **GS#-SUD** plate.

PCR Product

1.6b Pool 5 µL from each PCR reaction that is to be assayed be the same OPA. Transfer 5 µL of the pool to each well of the **GS#-SUD** plate.

1.7 Apply microplate foil seal, heat-seal the **GS#-SUD** plate, and pulse-centrifuge to 250 Xg.

1.8 Vortex at 2300 rpm for 20 seconds.

1.9 Pulse-centrifuge sealed plate to 250 Xg to prevent wells from drying out during the incubation.

1.11Heat **GS#-SUD** plate at 95°C for 30 min in preheated heat block, covering plate with heat block cover to reduce condensation (95°C incubation must not exceed 30 minutes).

1.12 Remove **GS#-SUD** plate from heat block and pulse-centrifuge to 250 Xg

(if proceeding to *Make ASE immediately after DNA preparation, immediately switch heat block to 70°C).*

2. Precip SUD (Pre-PCR Area)

This process precipitates the DNA in the GS#-SUD plate and removes excess DNA activation reagent GS#-MS1.

2.1. Carefully remove heat seal from **GS#-SUD** plate -- do not splash (use optional foil stripper).

2.2. Add 5 µL **GS#-PS1** reagent to each well of the **GS#-SUD** plate, and seal plate with adhesive film.

2.3. Pulse-centrifuge **GS#-SUD** plate to 250 Xg, then vortex at 2300 rpm for 20 seconds.

2.4. Remove adhesive film and add 15 µL 2-propanol to each well of **GS#-SUD** plate.

2.5. Seal **GS#-SUD** plate with adhesive film and vortex at 1600 rpm for 20 seconds.

2.6. Inspect wells for uniform blue color, and re-vortex if non-uniform appearance is observed.

2.7. Centrifuge sealed **GS#-SUD** plate at 3000 Xg for 20 minutes.

2.8. Remove **GS#-SUD** plate from centrifuge, remove plate seal, and decant supernatant by inversion onto an absorbent pad. Blot off excess supernatant.

2.9. Invert **GS#-SUD** plate on absorbent pad and centrifuge at 8 Xg for 1 minute. Do not spin the inverted plate at more than 8 Xg.

2.10.Allow plate to dry at room temperature for 15 minutes.

3. Resuspend SUD (Pre-PCR Area)

In this process, the DNA in the GS#-SUD plate is resuspended.

3.1. Pour 1.2 mL **GS#-RS1** into a sterile trough.

3.2. Add 10 µL **GS#-RS1** reagent to each well of **GS#-SUD** plate.

3.3. Seal **GS#-SUD** plate with adhesive film, pulse centrifuge to 250 Xg.

3.4. Vortex at 2300 rpm for 1 minute to resuspend blue pellet.

3.5. Verify that all blue pellets have been dissolved back into solution. If not, repeat vortex.

3.6. Either heat-seal the plate and store at 4°C, or proceed immediately to *Make ASE*.

N.B. For long-term storage, the activated DNA may be frozen at -20°C. If the activated DNA is stored frozen, thaw completely and vortex to mix contents before use in assay.

4. Make ASE (Pre-PCR Area)

This process combines the activated DNAs with SNP query oligos, hybridization reagents, and paramagnetic particles in an Allele Specific Extension (ASE) plate. The plate is then placed in a heat block and the allele specific and locus specific oligos for each SNP locus of interest are allowed to anneal to the activated DNA samples. The activated DNA is simultaneously captured by paramagnetic particles. The resulting GS#-ASE plate is ready for the extension and ligation of the hybridized oligos on the bound DNAs.

4.1. Remove **GS#-AM1** and **GS#-UB1** from refrigerator and thaw **GS#-MEL** to room temperature.

4.2. Pour 11 mL (+10 mL for each additional plate) **GS#-AM1** into sterile trough.

4.3. Pour 11 mL **GS#-UB1** into another sterile trough.

4.4. Centrifuge **GS#-ASE** plate to 250 Xg.

4.5. Place **GS#-ASE** plate on magnetic plate for ~ 2 minutes, or until beads are completely captured.

4.6. Remove heat seal from **GS#-ASE** plate; remove all liquid (~50 µL) from wells and discard liquid (leave beads in wells).

4.7.With **GS#-ASE** plate on magnetic plate, use new pipet tips and add 50 µL **GS#-AM1** to each well of **GS#-ASE** plate.

4.8.Seal **GS#-ASE** plate with adhesive film.

4.9.Vortex **GS#-ASE** plate at 1600 rpm for 20 seconds or until all beads are resuspended.

4.10.Place **GS#-ASE** plate on magnetic plate for ~2 minutes, or until beads are completely captured.

4.11.Using the same 8-channel pipet with the same tips, remove all **GS#-AM1** from each well.

4.12.Repeat addition of 50 µL **GS#-AM1**, vortexing, and removal of buffer.

4.13.Remove **GS#-ASE** plate from magnetic plate and, using ***new tips***, add 50 µL **GS#-UB1** to each well.

4.14.Place **GS#-ASE** plate onto magnetic plate for ~2 minutes, or until beads are completely captured.

4.15.Remove all **GS#-UB1** from each well (using same tips).

4.16.Repeat addition of 50 µL **GS#-UB1** and removal of buffer.

4.17.Pour thawed **GS#-MEL** reagent into a new sterile trough.

4.18.Using ***new pipet tips***, add 37 µL **GS#-MEL** to each well of **GS#-ASE** plate.

4.19.Seal plate with adhesive film, and vortex plate at 1600 rpm for 1 minute.

4.20.Incubate **GS#-ASE** plate on preheated 45°C heat block for 15 minutes.

5. Make PCR (Pre-PCR Area)

This process adds the Illumina-recommended DNA Polymerase and Uracil DNA Glycosylase (UDG -- OPTIONAL) to the master mix for PCR (GS#-MMP reagent) and creates a 96-sample plate for use in the Inoc PCR process.

5.1. Thaw one tube **GS#-MMP** to room temperature.
5.2. Add 64 µL Illumina-recommended DNA Polymerase to **GS#-MMP** tube.
5.3. Add 50 µL UDG to **GS#-MMP** tube (OPTIONAL).
5.4. Invert **GS#-MMP** tube several times to mix contents, and pour into a sterile trough.
5.5. Apply a **GS#-PCR** barcode label to a new 96-well 0.2 mL microplate.
5.6. Aliquot 30 µL of mixture into each well of **GS#-PCR** plate.
5.7. Seal **GS#-PCR** plate with clear adhesive film and pulse centrifuge to 250 Xg.

6. Inoc PCR (Pre-PCR Area)

This process uses the template formed in the extension and ligation process in a PCR reaction. This PCR reaction uses three universal primers: two are labeled with fluorescent dyes and the third is biotinylated. The biotinylated primer allows capture of the PCR product and elution of the strand containing the fluorescent signal.

6.1.Remove **GS#-ASE** plate from heat block
N.B. Do not transfer the plate to the 95°C heat block until all washes are completed!
6.2. Immediately preheat the heat block to 95°C.
6.3. Pour 6 mL **GS#-UB1** into a sterile trough.
6.4. Pour entire contents of **GS#-IP1** tube into another sterile trough.
6.5. Place **GS#-ASE** plate on magnetic plate for ~2 minutes, or until beads are captured.
6.6. Remove clear adhesive film from **GS#-ASE** plate.
6.7. Remove and discard supernatant (~50 µL) from all wells of the **GS#-ASE** plate (leave beads in wells).
6.8. Leave **GS#-ASE** plate on magnetic plate.
6.9. With ***new pipet tips***, add 50 µL **GS#-UB1** to each well of the **GS#-ASE** plate, and allow plate to rest on magnetic plate for at least 2 minutes.
6.10. Remove and discard all supernatant (~50 µL) from all wells of **GS#-ASE** plate (leave beads in wells).
6.11. Discard pipet tips.

If the normal GoldenGate protocol is being followed for a standard OPA than follow steps 6.12a-6.17a. If two standard oligo pools are to be merged follow steps 6.12b-6.17b. If a standard oligo pool is being merged with PCR product pools than follow steps 6.12c-6.17c.

GoldenGate Procedrue for a single OPA

6.12a. Add 35 µL **GS#-IP1** to each column of **GS#-ASE** plate and seal with adhesive film.
6.13a. Vortex at 1800 rpm for 1 minute, or until all beads are resuspended.
6.14a. Place the plate on 95°C heat block for 1 minute.
6.15a. Remove plate from heat block and place onto magnetic plate for 2 minutes, or until beads have settled completely.
6.16a. Transfer 30 µL supernatant from first column of **GS#-ASE** plate into first column of **GS#-PCR** plate, and discard pipet tips.
6.17a. Repeat transfer for each column of the **GS#-ASE** plate, using new pipet tips for each column.

Merging two Standard GoldengGate Oligo Pools

6.12b. Add 35 µL **GS#-IP1** to each column of **GS#-ASE-1 and GS#-ASE-2** plates and seal with adhesive film.
6.13b. Vortex at 1800 rpm for 1 minute, or until all beads are resuspended.
6.14b. Place the plates on 95°C heat block for 1 minute.
6.15b. Remove plates from heat block and place onto magnetic plate for 2 minutes, or until beads have settled completely.
6.16b. Transfer 15 µL supernatant from first column of **GS#-ASE-1** plate into first column of **GS#-PCR** plate, and discard pipet tips. Transfer 15 µL supernatant from first column of **GS#-ASE-2** plate into first column of **GS#-PCR** plate, and discard pipet tips.
6.17b. Repeat transfer for each column of the **GS#-ASE-1 and GS#-ASE-2** plates, using new pipet tips for each column.

Merging a standard oligo pool and a PCR product pool

6.12c. Add 35 µL **GS#-IP1** to each column of **GS#-ASE-1** (standard) **and GS#-ASE-2** (PCR) plate and seal with adhesive film.
6.13c. Vortex at 1800 rpm for 1 minute, or until all beads are resuspended.
6.14c. Place the plates on 95°C heat block for 1 minute.
6.15c. Remove plates from heat block and place onto magnetic plate for 2 minutes, or until beads have settled completely.
6.16c. Transfer 25 µL supernatant from first column of **GS#-ASE-1** plate into first column of **GS#-PCR** plate, and discard pipet tips. Transfer 5 µL supernatant from first column of **GS#-ASE-2** plate into first column of **GS#-PCR** plate, and discard pipet tips.

N.B. If there is more than one PCR ASE plate, than pool 30 µL of supernatant from each plate for each sample prior to transferring 5 µL to the **GS#-PCR** plate.

6.17c. Repeat transfer for each column of the **GS#-ASE-1 and GS#-ASE-2** plates, using new pipet tips for each column.

6.18. Seal **GS#-PCR** plate with Microseal "A" PCR plate sealing film.
6.19. Immediately transfer GS#-PCR plate to the thermal cycler immediately.
6.20. **GS#-ASE** plate may be discarded.

7.Complete PCR Steps -- Cycle PCR (Post-PCR Area)

This process thermal cycles the GS#-PCR plate to fluorescently label and amplify the templates generated in the pre-PCR process.

7.1.Place sealed plate into thermal cycler and run thermal cycler program (see Table 1)

Table 1. Thermal cycler program for GoldenGate

Temperature	Time at Temperature	
37°C	10 minutes	
95°C	3 minutes	
95°C	35 seconds	34 cycles
56°C	35 seconds	
72°C	2 minutes	
72°C	10 minutes	
4°C	5 minutes	

7.2. Proceed immediately to *Bind PCR*, or seal and store GS#-PCR plate at -20°C.

8.Bind PCR (Post-PCR Area)

In this step, the double-stranded PCR products are immobilized by binding the biotinylated strand to paramagnetic particles. Solution is transferred to a Filter plate and incubated at room temperature so that the PCR product may bind to the paramagnetic particles.

8.1.Vortex **GS#-MPB** tube until beads are completely resuspended.

8.2.Pour **GS#-MPB** into a sterile trough.

8.3.Write the PCR plate barcode number in the space provided on a

8.4.Apply the **Filter Plate: GS_____-PCR** label to the top surface of the **Filter plate** adjacent to column 12.
8.5.Pulse centrifuge **GS#-PCR** plate to 250 Xg.
8.6.Using ***new tips*** on a multi-channel pipet (5-50 µL), pipet 20 µL resuspended **GS#-MPB** from trough into each column of **GS#-PCR** plate. It is not necessary to change pipet tips again until liquid has been transferred to all 12 columns.

N.B. To avoid tip contamination, place the tips against the top edge of the wells. If you suspect the tips are contaminated with the contents of the well, discard the tips and use new tips.

8.7. Discard pipet tips.

8.8. Using ***new tips*** on a multi-channel pipet (50-300 µL) set to 85 µL, pipet solution in the first column of the **GS#-PCR** plate up and down several times to mix the beads with PCR product, then transfer the mixed solution to the first column of the **Filter plate**.

8.9. Using ***new pipet tips*** for each column, repeat the pipet mix and transfer step for the remaining columns of the **GS#-PCR** plate.

8.10. Discard the empty **GS#-PCR** plate.

8.11. Cover the **Filter plate** with its cover.

8.12. Store at room temperature, protected from light, for 60 minutes.

9.Make INT (Post-PCR Area)

In this step, the single-stranded fluor-labeled PCR product from the filter plate is washed and then eluted into an intermediate (INT) plate. The product from this plate is hybridized to the BeadChip.

9.1. Apply a **GS#-INT** barcode label to a new 96-well V-bottom plate.

9.2. Using a serological pipet, transfer 6 mL **GS#-UB2** into a sterile trough.

9.3. Pour 4 mL 0.1N NaOH into another sterile trough.

N.B. If you plan to proceed to hybridization immediately after making the INT plate, then begin resuspending the XC4 reagent now (see appendix 2: Resuspend XC4 Reagent).

9.4. When processing 8 BeadChips (96 samples), pour the GS#-MH1 tube's entire contents into a sterile trough. When any other combination of BeadChips are processed, aliquot 30 µL from the GS#-MH1 tube into each well.

9.5. Place **Filter plate** adapter on a new 96-well V-bottom Waste plate.

9.6. Place **Filter plate** containing the bound PCR products onto **Filter plate** adapter.

9.7. Centrifuge at 1000 Xg for 5 minutes at 25°C.

9.8. Remove **Filter plate** lid.

9.9. Using ***new pipet tips***, add 50 µL **GS#-UB2** from sterile trough to appropriate columns of the **Filter plate.**

N.B. To avoid disturbing the pellet or contaminating the tips, place the tips against the top edge of the wells.

9.10. Re-lid the **Filter plate** and centrifuge at 1000 Xg for 5 minutes at 25°C.

9.11. Usinga multichannel pipette with new tips, add 30 µl MH1 to each well containing sample of the INT plate.

9.12. Replace the waste plate with the INT plate. Orient the INT plate so that well A1 of the filter plate matches well A1 of the INT plate.

9.13. Discard the waste plate.

9.14. Using a multichannel pipette with new tips, add 30 µl 0.1N NaOH to each occupied well of the filter plate.

9.15. Replace the filter plate lid.

N.B. To avoid tip contamination, place the tips against the top edge of the wells. If you suspect the tips are contaminated with the contents of the well, discard the tips and use new tips.

9.16. Centrifuge immediately to 1000 xg for 5 minutes at 25°C. At the end, no beads should be visible in the wells of the INT plate.

9.17. Discard the filter plate. Save the adapter for later use in other protocols.

9.18. Gently mix the contents of the INT plate by moving it from side to side without splashing.

9.19. Seal the INT plate with a 96-well cap mat. Store the plate in the dark until ready to dispense sample onto a BeadChip.

10.Make HYB BeadChip (Post-PCR Area)

In this process, DNA samples from the INT plate are dispensed onto the BeadChips. The BeadChips are hybridized using the Hyb Chamber. After the Hyb Chamber has been assembled, the DNA-loaded BeadChips are placed into the Hyb Chamber and the samples are ready for hybridization. The BeadChip is hybridized overnight in the Illumina Hybridization Oven, with a temperature ramp-down from 60°C to 45°C.

10.1.Preheat oven to 60°C and allow oven to equilibrate.

10.2.Place the following items on the bench top:

• BeadChip Hyb Chamber (1 per 4 BeadChips)
• BeadChip Hyb Chamber gasket (1 per Hyb Chamber)
• BeadChip Hyb Chamber inserts (4 per Hyb Chamber)

10.3.Place the Hyb Chamber Gasket into the Hyb Chamber.

10.3.1 Match the wider edge of the Hyb Chamber gasket to the barcode-ridge side of the Hyb Chamber.

10.3.2 Lay the gasket into the Hyb Chamber and then press it down all around

10.3.3 Make sure the Hyb Chamber gasket is properly seated.

10.4.Add 200 µl CHB into each of the eight humidifying buffer reservoirs in the Hyb Chamber. If you are hybridizing fewer than four BeadChips, only fill the reservoirs of sections that will contain BeadChips.

10.5. Close and lock the BeadChip Hyb Chamber lid

10.5.1 Seat the lid securely on the bottom plate

10.5.2 Snap two clamps shut, diagonally across from each other.

10.5.3 Snap the other two clamps.

10.6.Leave the closed Hyb Chamber on the bench at room temperature (22°C) until the BeadChips are loaded with DNA sample.

11. Prepare BeadChip for HYB (Post-PCR Area)

11.1.Remove all the BeadChips from their packages.

11.2.Place each BeadChip in a Hyb Chamber Insert, orienting the barcode end so that it matches the barcode symbol on the Hyb Chamber Insert.

12.Load Sample (Post-PCR Area)

12.1.Pipette samples up and down in the INT plate to mix.

12.2.Using a single or multi-channel precision pipette, add 15 µl sample from the INT plate onto the center of each inlet port.

12.3.Visually inspect all sections. Ensure sample covers all of the sections of the stripe. Record any sections that are not covered on the lab tracking worksheet.

12.4.Open the Hyb Chamber.

12.5. Load four Hyb Chamber Inserts containing sample-laden BeadChips into each Hyb Chamber

12.6.Position the barcode end over the ridges indicated on the Hyb Chamber and ensure the inserts are securely seated.

12.7.Close and lock the BeadChip Hyb Chamber lid.

12.7.1 Seat the lid securely on the bottom plate

12.7.2 Snap two clamps shut, diagonally across from each other.

12.7.3 Snap the other two clamps.

13.Hybridize BeadChips(Post-PCR Area)

13.1. Place the Hyb Chamber into the 60°C Illumina Hybridization Oven so that the clamps face the left and right sides of the oven. The Illumina logo on top of the Hyb Chamber should face you.

13.2.Incubate for exactly 30 minutes at 60ºC.

13.3.After 30 minutes, reset the temperature to 45ºC.

13.4.Incubate for at least 14 hours but no more than 20 hours at 45°C.

14. Wash and Coat BeadChip (Post-PCR Area)

In this process, the BeadChips are removed from the Hyb Chamber and washed three times with UB2 and XC4 reagents. There are two separate UB2 washes and one XC4 wash. To process multiple BeadChips in parallel, set up a group of wash stations for each wash rack filled with BeadChips. Start washing subsequent BeadChips while the previous ones are incubating in UB2 or XC4.

14.1. Set up three top-loading wash dishes, labeled UB2, UB2 and XC4.

14.2.Submerge the unloaded wash rack into the first UB2 wash dish with the locking arms facing you. This orients the wash rack so that you can safely remove the BeadChips.

14.3.With a hand on top of the Hyb Chamber, un-snap the four clips one at a time. Start with the first clip, then un-snap the clip that is diagonally across from it and so on. Lift the lid straight up and off. Set the lid to the side on the bench.

14.4.Remove each Hyb Chamber from the Illumina Hybridization Oven.

14.5.Remove the first BeadChip from a Hyb Chamber insert.

14.6.Remove the IntelliHyb Seal from the BeadChip:

14.6.1 Wearing powder-free gloves, hold the BeadChip in one hand with your thumb and forefinger on opposing edges of the BeadChip. Do not touch the sample inlets. The barcode should face up and be closest to you, and the top side of the BeadChip should be angled slightly away from you.

14.6.2 Remove the entire seal in a single, rapid motion by pulling it off in a diagonal direction. Start with a corner on the barcode end and pull with a continuous upward motion away from you and towards the opposite corner on the top side of the BeadChip. Do not stop and start the pulling action. Do not touch the exposed active areas.

14.6.3 Discard the seal.

14.7.Holding the BeadChip by the barcode end, immediately and carefully slide the BeadChip into the wash rack while it is submerged in UB2.

14.8.Ensure that the BeadChip barcodes are correctly positioned in the wash rack, with the labels facing up and away from you. This is essential for proper handling and coating.

14.9.Repeat steps 14. 5–14.8 until all BeadChips are transferred to the submerged wash rack. Load the wash rack with up to 8 BeadChips. Put four BeadChips above the wash rack handle and four below.

14.10. Once all BeadChips are in the wash rack, move the wash rack up and down ten times, breaking the surface of the UB2 with gentle, slow agitation.

14.11. Transfer the wash rack to the second UB2 wash dish and let it soak for five minutes.

14.12 Transfer the wash rack to the XC4 wash dish and slowly move the wash rack up and down ten times. Let it soak for five minutes. The barcode labels on the BeadChips must face away from you, while the locking arms on the handle face towards you, for proper handling and coating.

15. Dry BeadChips (Post-PCR Area)

15.1.Prepare a clean tube rack for the wash rack by placing two folded Kimwipes under the tube rack.

15.2.Prepare one additional tube rack per 8 BeadChips that fits the internal dimensions of the vacuum desiccator.

15.3.Remove the wash rack in one smooth, rapid motion and place it directly on the prepared tube rack, making sure the barcodes face *up* and the locking arms and tab face *down.*

15.4.For the top four BeadChips, working top to bottom:

15.4.1 Continuing to hold the wash rack handle, carefully grip each BeadChip at its barcode end with self-locking tweezers.

15.4.2 Place the BeadChip on a tube rack with the barcode facing up and towards you.

15.5.Holding the top of the wash rack in position, gently remove the wash rack handle by grasping the handle between the thumb and forefinger. Push the tab up with your thumb

and push the handle away from you (unlocking the handle), then pull up the handle and remove.

15.6. Place any remaining BeadChips on the tube rack with six BeadChips on top of the rack and two BeadChips on the bottom. The barcode ends should be towards you, and the BeadChips should be completely horizontal.

15.7. Place the tube rack in the vacuum desiccator. Each dessicator can hold one tube rack (8 BeadChips).

15.8. Ensure the vacuum valve is seated tightly and securely.

15.9. Start the vacuum, using at least 508 mm Hg (0.68 bar).

15.10. To ensure that the dessicator is properly sealed, gently lift the lid of the vacuum desiccator. It should not lift off the desiccator base.

15.11. Dry under vacuum for 50–55 minutes. (Drying times may vary according to room temperature and humidity)

15.12. Release the vacuum by turning the handle very slowly.

15.13. Touch the borders of the chips (*do not touch the stripes*) to ensure that the etched, bar-coded side of the BeadChips are dry to the touch.

15.14. If the underside feels tacky, manually clean the underside of the BeadChip to remove any excess XC4. The bottom two BeadChips are the most likely to have some excess.

15.14.1 Wrap a pre-saturated Prostat EtOH Wipe around your index finger.

15.14.2 Wipe along the underside of the BeadChip five or six times, until the surface is clean and smooth.

15.15. Clean the Hyb Chambers:

15.15.1 Remove the rubber gaskets from the Hyb Chambers.

15.15.2 Rinse all Hyb Chamber components with DI water.

15.15.3 Thoroughly rinse the eight humidifying buffer reservoirs.

15.16. Clean and dry the wash dishes:

15.16.1 Rinse the UB2 wash dishes with DI water.

15.16.2 Rinse the XC4 wash dish with ethanol.

15.16.3 Air dry all wash dishes.

15.17. Discard unused reagents in accordance with facility standards.

15.18. Proceed to *Image BeadChip on the iScan System*.

16.BeadChips Imaging

The iScan Reader uses a laser to excite the fluor of the single-base extension product on the beads of the BeadChip sections. Light emissions from these fluors are then recorded in high-resolution images of the BeadChip sections. Data from these images are analyzed using Illumina's GenomeScan software.

16.1. Turn on the iScan Reader, boot up the iScan PC, and start the GenomeScan application.

N.B. Turn on the iScan Reader before launching the GenomeScan software. If the software is launched when the instrument is turned off, an error message will alert you that the hardware is missing.

16.2. Load the BeadChips to be scanned, and copy their decode data into the Input Path.

16.3. Check the scan settings and input/output paths, making modifications if necessary.

16.4.If you wish, remove BeadChip sections or entire BeadChips from the scan.
16.5.Start the scan and monitor its progress.
16.6.Review the scan metrics.

17. Reference Documents

17.1. Illumina, BeadStation500G, Experienced User Cards, Rev B

17.2. Illumina, BeadStation500G, System Manual, RevB

17.3. Illumina, Universal 12 BeadChip, Booklet, Rev A1

Appendix 3: Final Gene and SNP list for the ADME Panel:

OPA 1				OPA 2			
Gene/Loci	**RS**	**Chr**	**Coordinate**	**Gene/Loci**	**RS**	**Chr**	**Coordinate**
ALDH4A1	rs9117	1	19071482		rs10864327	1	8077134
ALDH4A1	rs6664725	1	19075814	CYP4B1	rs7540241	1	47030806
ALDH4A1	rs7365362	1	19083403	CYP4B1	rs6659627	1	47094286
ALDH4A1	rs4912075	1	19088005	GPX7	rs7527068	1	52849213
ALDH4A1	rs1009971	1	19094607	CYP2J2	rs12092576	1	60082886
ALDH4A1	rs4912044	1	19102850	CYP2J2	rs2294950	1	60087084
ALDH4A1	rs6673711	1	19108104	CYP2J2	rs3754203	1	60096541
ALDH4A1	rs16862355	1	19108245		rs11162555	1	79032440
ALDH4A1	rs16862363	1	19111529	ABCA4	rs7518454	1	94235954
CDA	rs635479	1	20785381	ABCA4	rs10874828	1	94242161
CDA	rs603412	1	20788005	ABCA4	rs3789387	1	94247549
CDA	rs818202	1	20789378	ABCA4	rs1801574	1	94248976
CDA	rs471760	1	20789793	ABCA4	rs537831	1	94249143
CDA	rs10916824	1	20791499	ABCA4	rs11165063	1	94250237
CDA	rs818196	1	20799218	ABCA4	rs945067	1	94250481
CDA	rs517483	1	20802622	ABCA4	rs17391542	1	94250523
CDA	rs818194	1	20804415	ABCA4	rs12083701	1	94252313
CDA	rs10916827	1	20805928	ABCA4	rs915200	1	94254492
CDA	rs580032	1	20806480	ABCA4	rs17110858	1	94258943
CDA	rs1689924	1	20807383	ABCA4	rs2282229	1	94260914
CDA	rs12404655	1	20815782	ABCA4	rs17110885	1	94265819
CDA	rs1614627	1	20819343	ABCA4	rs17392369	1	94284948
CDA	rs10916837	1	20827770	ABCA4	rs12123388	1	94285689
CDA	rs10916843	1	20850724	ABCA4	rs6658767	1	94302483
CYP4B1	rs863915	1	47031725	ABCA4	rs10493868	1	94304146
CYP4B1	rs632233	1	47036792	ABCA4	rs7366102	1	94304850
CYP4B1	rs3766209	1	47037860	ABCA4	rs2151848	1	94310148
CYP4B1	rs837398	1	47039009	ABCA4	rs17461953	1	94324038
CYP4B1	rs837395	1	47041925	ABCA4	rs12088309	1	94336504
CYP4B1	rs4646481	1	47044125	ABCA4	rs548122	1	94338018
CYP4B1	rs4646485	1	47051426	ABCA4	rs35146614	1	94342777
CYP4B1	rs4646486	1	47051584	ABCA4	rs1191236	1	94344858
CYP4B1	rs13373822	1	47062500	ABCA4	rs4147798	1	94357597
GPX7	rs835344	1	52823608	ABCA4	rs11165081	1	94366668
GPX7	rs835337	1	52831694	DPYD	RS1801268	1	97317215
GPX7	rs6588431	1	52840174	DPYD	RS1801267	1	97336742
GPX7	rs946154	1	52845915	DPYD	rs1801266	1	97929920
GPX7	rs7529595	1	52849251	GSTM1/2/3/4/5	rs542338	1	109994289
GPX7	rs6671552	1	52854173	GSTM1/2/3/4/5	rs1010167	1	110000250

GPX7	rs11581211	1	52863509
CYP2J2	rs11207530	1	60111364
CYP2J2	rs10789082	1	60130557
CYP2J2	rs1155002	1	60146363
CYP2J2	rs10493270	1	60161262
CYP2J2	rs12731852	1	60181563
ABCA4	rs1889547	1	94222719
ABCA4	rs3747961	1	94231009
ABCA4	rs1932016	1	94232931
ABCA4	rs6666652	1	94234305
ABCA4	rs3789375	1	94237720
ABCA4	rs4147871	1	94238049
ABCA4	rs4847195	1	94241396
ABCA4	rs7537325	1	94242219
ABCA4	rs2275031	1	94246484
ABCA4	rs1191234	1	94246608
ABCA4	rs17110808	1	94247460
ABCA4	rs12085639	1	94250881
ABCA4	rs2275033	1	94252625
ABCA4	rs3818778	1	94253117
ABCA4	rs914958	1	94253656
ABCA4	rs915199	1	94254517
ABCA4	rs17110850	1	94258079
ABCA4	rs10493867	1	94258994
ABCA4	rs4147848	1	94259175
ABCA4	rs472908	1	94259942
ABCA4	rs1932014	1	94261085
ABCA4	rs11165065	1	94264056
ABCA4	rs4147844	1	94268075
ABCA4	rs3789395	1	94274182
ABCA4	rs1889548	1	94275785
ABCA4	rs11165069	1	94277133
ABCA4	rs4147839	1	94281814
ABCA4	rs484110	1	94287545
ABCA4	rs497511	1	94295701
ABCA4	rs549848	1	94297444
ABCA4	rs1007347	1	94303106
ABCA4	rs1191232	1	94303780
ABCA4	rs3789407	1	94304194
ABCA4	rs1931575	1	94305602
ABCA4	rs549114	1	94306942
ABCA4	rs4612636	1	94308277
ABCA4	rs3789412	1	94308655
GSTM1/2/3/4/5	rs625456	1	110012194
GSTM1/2/3/4/5	rs673151	1	110014981
GSTM1/2/3/4/5	rs574344	1	110015037
GSTM1/2/3/4/5	rs655315	1	110016701
GSTM1/2/3/4/5	rs12024479	1	110021609
GSTM1/2/3/4/5	rs366732	1	110024530
GSTM1/2/3/4/5	rs412543	1	110031467
GSTM1	rs1065411	1	110034661
GSTM1/2/3/4/5	rs1292099	1	110048687
GSTM1/2/3/4/5	rs11579576	1	110050699
GSTM1/2/3/4/5	rs674787	1	110055434
GSTM1/2/3/4/5	rs1758104	1	110056201
GSTM1/2/3/4/5	rs12736389	1	110056690
GSTM1/2/3/4/5	rs886177	1	110058030
GSTM1/2/3/4/5	rs12743015	1	110058454
GSTM1/2/3/4/5	rs3768490	1	110060539
GSTM1/2/3/4/5	rs4970776	1	110072980
GSTM1/2/3/4/5	rs12059276	1	110075064
GSTM1/2/3/4/5	rs15864	1	110094379
SLC16A1	rs12727968	1	113253807
SLC16A1	rs4839273	1	113304075
SLC16A1	rs17494145	1	113348865
SLC22A15	rs10733105	1	116313417
SLC22A15	rs1281693	1	116319842
SLC22A15	rs2486081	1	116338170
SLC22A15	rs6667257	1	116348182
SLC22A15	rs12023924	1	116367313
SLC22A15	rs12562630	1	116372348
SLC22A15	rs17515267	1	116407661
SLC22A15	rs12410137	1	116421214
FMO5	rs10900321	1	145096540
FMO5	rs12401360	1	145127071
FMO5	rs718369	1	145135518
FMO5	rs10494242	1	145137254
FMO5	rs17159924	1	145137668
FMO5	rs894469	1	145139530
FMO5	rs2297753	1	145150755
FMO5	rs2354432	1	145159853
FMO5	rs12140294	1	145175208
FMO5	rs7537483	1	145176501
ARNT	rs10888395	1	149028795
ARNT	rs4379678	1	149047206
ARNT	rs10305724	1	149061989

ABCA4	rs12759306	1	94310230
ABCA4	rs524322	1	94313912
ABCA4	rs17111003	1	94314437
ABCA4	rs3120133	1	94315358
ABCA4	rs4147830	1	94316864
ABCA4	rs560426	1	94326026
ABCA4	rs4847196	1	94327041
ABCA4	rs1191238	1	94329482
ABCA4	rs554931	1	94329945
ABCA4	rs952499	1	94331013
ABCA4	rs2068334	1	94332303
ABCA4	rs4147825	1	94333526
ABCA4	rs4147822	1	94334077
ABCA4	rs4147820	1	94334672
ABCA4	rs3789421	1	94338165
ABCA4	rs950283	1	94339811
ABCA4	rs570926	1	94342806
ABCA4	rs4147815	1	94347396
ABCA4	rs3789433	1	94348028
ABCA4	rs2297634	1	94349556
ABCA4	rs1889404	1	94350011
ABCA4	rs4147807	1	94351641
ABCA4	rs10782976	1	94353713
ABCA4	rs3789441	1	94353972
ABCA4	rs3761906	1	94359950
ABCA4	rs3789452	1	94360089
ABCA4	rs2151846	1	94360275
ABCA4	rs1105123	1	94364878
ABCA4	rs17111122	1	94369052
DPYD	rs10875044	1	97291251
DPYD	rs17430460	1	97315067
DPYD	rs1042482	1	97316547
DPYD	rs1023244	1	97318788
DPYD	rs290852	1	97322779
DPYD	rs17471125	1	97325716
DPYD	rs2056048	1	97330750
DPYD	rs10449721	1	97332311
DPYD	rs11165779	1	97337238
DPYD	rs11165783	1	97342132
DPYD	rs17471577	1	97352799
DPYD	rs6681958	1	97356749
DPYD	rs9437663	1	97358525
DPYD	rs4950021	1	97366648
ARNT	rs10305710	1	149069651
ARNT	rs10305695	1	149075740
NR1I3	rs5082	1	159460307
NR1I3	rs11265571	1	159478523
ALDH9A1	rs7527279	1	163888214
ALDH9A1	rs4147602	1	163888402
FMO3	rs10910879	1	169319777
FMO3	rs16863990	1	169322194
FMO3	rs1736560	1	169325774
FMO3	rs2075992	1	169347109
FMO3	rs12080672	1	169354339
FMO3	rs742057	1	169357608
FMO3	rs2064080	1	169369865
FMO1/2	rs2307492	1	169435169
	rs11587539	1	185181800
EPHX1	rs17571067	1	224070785
EPHX1	rs10753410	1	224074724
EPHX1	rs2854450	1	224079200
EPHX1	rs1051740	1	224086256
EPHX1	rs2260863	1	224086397
EPHX1	rs2740168	1	224087611
EPHX1	rs10915884	1	224090498
EPHX1	rs2671267	1	224092313
EPHX1	rs6965	1	224100099
CYP1B1	rs4670235	2	38126032
CYP1B1	rs162561	2	38152381
	rs2900805	2	71311975
	rs10189601	2	104568129
SULT1C1	rs2305485	2	108271480
SULT1C1	rs12998619	2	108272846
SULT1C1	rs2198466	2	108293399
SULT1C1	rs752273	2	108297537
SULT1C2	rs13021399	2	108373097
	rs4988235	2	136325116
	rs13395686	2	136571458
	rs3911925	2	162116176
	rs2389550	2	162166647
ABCB11	rs504393	2	169439579
ABCB11	rs569805	2	169491126
ABCB11	rs16823014	2	169525959
ABCB11	rs4148794	2	169529894
ABCB11	rs4233823	2	169599137
ABCB11	rs7577650	2	169599456

DPYD	rs17431828	1	97372898
DPYD	rs1709409	1	97375314
DPYD	rs7552825	1	97379352
DPYD	rs6667550	1	97379808
DPYD	rs17702702	1	97380425
DPYD	rs828053	1	97381914
DPYD	rs828054	1	97382614
DPYD	rs1760215	1	97384103
DPYD	rs1514495	1	97390377
DPYD	rs12133667	1	97409312
DPYD	rs11165798	1	97411138
DPYD	rs12035560	1	97414359
DPYD	rs6661442	1	97416050
DPYD	rs6656660	1	97421797
DPYD	rs11587873	1	97425658
DPYD	rs1413228	1	97433280
DPYD	rs6593639	1	97442913
DPYD	rs12120068	1	97452904
DPYD	rs1333727	1	97453930
DPYD	rs12046655	1	97454964
DPYD	rs4415625	1	97461419
DPYD	rs885622	1	97463593
DPYD	rs676686	1	97471225
DPYD	rs11165814	1	97480849
DPYD	rs12124488	1	97481641
DPYD	rs12758854	1	97484149
DPYD	rs10875057	1	97487253
DPYD	rs12069661	1	97487743
DPYD	rs12735722	1	97492522
DPYD	rs1413241	1	97495260
DPYD	rs2027055	1	97495652
DPYD	rs2027056	1	97496189
DPYD	rs12140120	1	97496597
DPYD	rs11165823	1	97498267
DPYD	rs499009	1	97503990
DPYD	rs12048612	1	97506520
DPYD	rs7539889	1	97513711
DPYD	rs667565	1	97521836
DPYD	rs11165837	1	97531608
DPYD	rs4264037	1	97536661
DPYD	rs7555294	1	97572133
DPYD	rs9782950	1	97576312
DPYD	rs11588048	1	97584162
	rs12624292	2	172533589
	rs1011584	2	190606463
	rs10084389	2	213834442
	rs691968	2	219382394
ABCB6	rs4674367	2	219781096
ABCB6	rs17783626	2	219782686
ABCB6	rs908196	2	219796492
UGT1A	rs12467878	2	234145095
UGT1A	rs2741019	2	234175427
UGT1A	rs4402757	2	234181194
UGT1A	rs6431558	2	234194382
UGT1A	rs1597942	2	234200589
UGT1A	rs4663871	2	234246326
UGT1A	rs28948388	2	234251257
UGT1A	rs6751673	2	234269642
UGT1A	rs6715829	2	234270901
UGT1A	rs4233633	2	234284676
UGT1A	rs6755571	2	234292275
UGT1A	rs2011404	2	234292676
UGT1A	rs4663964	2	234314975
UGT1A	rs13009407	2	234317086
UGT1A1	rs35350960	2	234334358
UGT1A	rs28946889	2	234336201
UGT1A1	rs55750087	2	234341619
UGT1A1	rs34993780	2	234345798
UGT1A	rs10203853	2	234352157
UGT1A	rs6431632	2	234355284
UGT1A	rs6431633	2	234355530
PPARG	rs2920502	3	12304195
PPARG	rs12493718	3	12338637
PPARG	rs17793951	3	12345737
PPARG	rs10510418	3	12363563
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PPARG	rs1801282	3	12368125
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PPARG	rs4135284	3	12424866
PPARG	rs4135292	3	12440956
PPARG	rs6782475	3	12446330
PPARG	rs1152003	3	12452055
PPARG	rs9833097	3	12453817
PPARG	rs709159	3	12456203

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	rs12705915	7	113260505
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	rs10260295	7	151838242
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ADH1A/B/C	rs2173199	4	100390402
ADH6	rs7684914	4	100393567
ADH1A/B/C	rs1497379	4	100395093
SLCO2B1	rs1676885	11	74582926
SLCO2B1	rs1789690	11	74603176
NNMT	rs11214942	11	113695587
NNMT	rs2852439	11	113697310
SLCO1B3/1C1	rs7975334	12	20731118
SLCO1B3/1C1	rs35653399	12	20767250
SLCO1B3/1C1	rs12820079	12	20767494
SLCO1B3/1C1	rs7310077	12	20788391
SLCO1B3/1C1	rs4581504	12	20790983
SLCO1B3/1C1	rs972507	12	20794787
SLCO1B3/1C1	rs7977213	12	20872067
SLCO1B3/1C1	rs11045577	12	20920465
SLCO1B3/1C1	rs10841697	12	20947477
SLCO1B3/1C1	rs1865159	12	20962526
SLCO1B3/1C1	rs11045611	12	20990539
SLCO1B1/1A2	rs16923519	12	21202985
SLCO1B1/1A2	rs7138177	12	21204191
SLCO1B1/1A2	rs7953338	12	21205839
SLCO1B1/1A2	rs4149032	12	21209058
SLCO1B1	rs56101265	12	21216983
SLCO1B1	rs56061388	12	21218796
SLCO1B1	RS4149056	12	21222816
SLCO1B1/1A2	rs4149058	12	21224481
SLCO1B1/1A2	rs991262	12	21225481
SLCO1B1	rs55901008	12	21244796
SLCO1B1	rs59502379	12	21250200
SLCO1B1/1A2	rs4149070	12	21261150
SLCO1B1/1A2	rs7137060	12	21274097
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SLCO1B1/1A2	rs7960688	12	21287136
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SLCO1B1/1A2	rs7966334	12	21358482
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SLCO1B1/1A2	rs17681288	12	21436279
SLCO1B1/1A2	rs7316461	12	21437894
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	rs12018394	13	19867210
	rs945177	13	26519985
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ABCC1/6	rs7186601	16	16196257
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ABCC1/6	rs4781735	16	16197743
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SULT1A1	rs1801030	16	28524986
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SLC22A18	rs2283231	11	2873291
SLC22A18	rs365605	11	2877242
SLC22A18	rs367035	11	2880402
SLC22A18	rs413781	11	2882197
SLC22A18	rs389646	11	2882337
SLC22A18	rs4930028	11	2889641
SLC22A18	rs3782067	11	2891879
SLC22A18	rs3764895	11	2902521
ABCC8	rs2285676	11	17364601
ABCC8	rs5215	11	17365206
ABCC8	rs8192690	11	17371146
ABCC8	rs8192691	11	17373913
ABCC8	rs739689	11	17374080
ABCC8	rs1799859	11	17375855
ABCC8	rs2074311	11	17378436
ABCC8	rs12422139	11	17378973
ABCC8	rs2077654	11	17384477
ABCC8	rs7926934	11	17389664
ABCC8	rs2355017	11	17391179
ABCC8	rs2074309	11	17391790
ABCC8	rs2237991	11	17396805
ABCC8	rs916829	11	17397049
ABCC8	rs2299639	11	17397961
ABCC8	rs929235	11	17399036
ABCC8	rs2237988	11	17400710
ABCC8	rs2237984	11	17402597
ABCC8	rs2074308	11	17409156
ABCC8	rs4148618	11	17410019
ABCC8	rs11024286	11	17415683
ABCC8	rs7950189	11	17424893
ABCC8	rs722341	11	17429722
ABCC8	rs1048099	11	17453092
ABCC8	rs3758949	11	17457639
ABCC8	rs12278956	11	17464789
SLC22A6/8	rs6591722	11	62506256
SLC22A6/8	rs10897312	11	62508234
SLC22A6/8	rs955434	11	62513689
SLC22A6/8	rs4149183	11	62522445
SLC22A6/8	rs2276299	11	62523007
SLC22A6/8	rs2187383	11	62532474
SLC22A6/8	rs948979	11	62540402
SLC22A6/8	rs10792369	11	62543011
SLC13A3	rs4810535	20	44682123
SLC13A3	rs742594	20	44690385
SLC13A3	rs16991650	20	44691134
SLC13A3	rs2425884	20	44691699
SLC13A3	rs3091520	20	44692047
SLC13A3	rs2425885	20	44693038
SLC13A3	rs2425886	20	44693250
SLC13A3	rs941206	20	44694448
SLC13A3	rs2425887	20	44695530
SLC13A3	rs3091556	20	44696189
SLC13A3	rs17481569	20	44698593
SLC13A3	rs2694903	20	44700354
SLC13A3	rs6017949	20	44702963
SLC13A3	rs11699754	20	44707758
SLC13A3	rs6090536	20	44708393
SLC13A3	rs2757524	20	44709070
SLC13A3	rs11907381	20	44710186
SLC13A3	rs1004571	20	44711576
SLC13A3	rs2757523	20	44715003
SLC13A3	rs6066043	20	44721860
SLC13A3	rs13038390	20	44726581
CYP24A1	rs2762920	20	52183230
CYP24A1	rs873216	20	52192413
CYP24A1	rs2762926	20	52195825
CYP24A1	rs2585413	20	52195887
CYP24A1	rs2762927	20	52196373
CYP24A1	rs2031343	20	52197270
CYP24A1	rs6022985	20	52199020
CYP24A1	rs2762929	20	52199602
CYP24A1	rs8124792	20	52200214
CYP24A1	rs6068810	20	52202758
CYP24A1	rs6097807	20	52202862
CYP24A1	rs2762933	20	52203310
CYP24A1	rs4809957	20	52204578
CYP24A1	rs2762934	20	52204668
CYP24A1	rs927650	20	52206148
CYP24A1	rs1570669	20	52207834
CYP24A1	rs2209314	20	52212368
CYP24A1	rs6127118	20	52212459
CYP24A1	rs6068816	20	52214498
CYP24A1	rs2762939	20	52214658
CYP24A1	rs3787554	20	52216087
CYP24A1	rs2244719	20	52216265

SLC22A6/8	rs10897315	11	62545707
SLC22A6/8	rs10897316	11	62546339
SLC22A9	rs556730	11	62899453
SLC22A9	rs604285	11	62925353
SLC22A9	rs630759	11	62933285
SLC22A11	rs693591	11	64081645
SLC22A11	rs1783811	11	64089872
SLC22A12	rs475688	11	64120867
SLC22A12	rs17146121	11	64143102
GSTP1	rs612020	11	67102017
GSTP1	rs614080	11	67103863
GSTP1	rs1695	11	67109265
ALDH3B2	rs10736660	11	67176431
ALDH3B2	rs1551886	11	67187338
ALDH3B2	rs11603033	11	67205316
ALDH3B1	rs11228120	11	67525372
ALDH3B1	rs12418774	11	67530807
ALDH3B1	rs479763	11	67531704
ALDH3B1	rs3763941	11	67532938
ALDH3B1	rs1003777	11	67548543
ALDH3B1	rs105147	11	67549590
ALDH3B1	rs3794176	11	67551219
ALDH3B1	rs4147779	11	67561383
ALDH3B1	rs884826	11	67562705
SLCO2B1	rs4944991	11	74538434
SLCO2B1	rs2851071	11	74543364
SLCO2B1	rs4944993	11	74543488
SLCO2B1	rs1789694	11	74559330
SLCO2B1	rs12422149	11	74561225
SLCO2B1	rs7947726	11	74563722
SLCO2B1	rs1676881	11	74564506
SLCO2B1	rs11823030	11	74584852
SLCO2B1	rs7116044	11	74589602
NNMT	rs2244175	11	113667731
NNMT	rs2852432	11	113669214
NNMT	rs4646335	11	113672152
NNMT	rs3819100	11	113672686
NNMT	rs2301128	11	113673209
NNMT	rs1941404	11	113674248
NNMT	rs10891645	11	113676457
NNMT	rs2155806	11	113677720
NNMT	rs1941398	11	113683266
NNMT	rs949370	11	113693674
CYP24A1	rs3787557	20	52216542
CYP24A1	rs2762941	20	52217059
CYP24A1	rs2181874	20	52217885
CYP24A1	rs4809959	20	52219266
CYP24A1	rs4809960	20	52219480
CYP24A1	rs2245153	20	52219813
CYP24A1	rs13038432	20	52220709
CYP24A1	rs6022999	20	52221420
CYP24A1	rs2248359	20	52224925
CYP24A1	rs2426498	20	52230088
CYP24A1	rs2208588	20	52230378
CYP24A1	rs2585424	20	52231986
CYP24A1	rs6023012	20	52239980
SLCO4A1	rs6089917	20	60734665
SLCO4A1	rs6062918	20	60741383
SLCO4A1	rs2427364	20	60751170
SLCO4A1	rs17311216	20	60757617
SLCO4A1	rs3195701	20	60758800
SLCO4A1	rs2236553	20	60760188
SLCO4A1	rs2258022	20	60769411
SLCO4A1	rs12481701	20	60772300
SLCO4A1	rs6062771	20	60772955
SLCO4A1	rs3787538	20	60773449
SLCO4A1	rs1055196	20	60774011
SLCO4A1	rs3787537	20	60774187
SLCO4A1	rs6010817	20	60774260
SLCO4A1	rs13037524	20	60775645
SLCO4A1	rs2427372	20	60775725
SLCO4A1	rs6062400	20	60776381
SLCO4A1	rs4809480	20	60777875
SLCO4A1	rs7266121	20	60780515
SLCO4A1	rs2427374	20	60781847
SLCO4A1	rs2313152	20	60782438
SLCO4A1	rs2427377	20	60784589
SLCO4A1	rs2427378	20	60784723
SLCO4A1	rs2105161	20	60788376
	rs6011368	20	62356948
CBR1	rs2835235	21	36319470
CBR1	rs7275820	21	36331284
CBR1	rs1005696	21	36365350
CBR1	rs9024	21	36367183
CBR1	rs4239798	21	36368322
CBR1	rs2835268	21	36371006

NNMT	rs1941396	11	113695631
NNMT	rs7929497	11	113697342
NNMT	rs11214958	11	113717478
SLCO1B3/1C1	rs3809208	12	20739263
SLCO1B3/1C1	rs10770704	12	20747028
SLCO1B3/1C1	rs11045399	12	20754206
SLCO1B3/1C1	rs1515777	12	20754840
SLCO1B3/1C1	rs952658	12	20756568
SLCO1B3/1C1	rs10841596	12	20759021
SLCO1B3/1C1	rs2056136	12	20759160
SLCO1B3/1C1	rs2417862	12	20761532
SLCO1B3/1C1	rs2203494	12	20766653
SLCO1B3/1C1	rs16923154	12	20777198
SLCO1B3/1C1	rs974453	12	20781209
SLCO1B3/1C1	rs17379695	12	20794062
SLCO1B3/1C1	rs11045505	12	20840713
SLCO1B3/1C1	rs1515766	12	20854402
SLCO1B3/1C1	rs12228798	12	20855824
SLCO1B3/1C1	rs7310629	12	20876554
SLCO1B3/1C1	rs2900474	12	20901315
SLCO1B3	rs4149117	12	20902747
SLCO1B3	rs7311358	12	20907027
SLCO1B3/1C1	rs3764006	12	20945636
SLCO1B3/1C1	rs2117032	12	20965389
SLCO1B1/1A2	rs704166	12	21171109
SLCO1B1/1A2	rs3829307	12	21183473
SLCO1B1/1A2	rs2417954	12	21187611
SLCO1B1/1A2	rs7489119	12	21196565
SLCO1B1/1A2	rs4149023	12	21200724
SLCO1B1/1A2	rs4149026	12	21206682
SLCO1B1/1A2	rs4149033	12	21209077
SLCO1B1/1A2	rs4149035	12	21209532
SLCO1B1/1A2	rs2291073	12	21217081
SLCO1B1/1A2	rs964614	12	21220657
SLCO1B1	RS2306283	12	21221005
SLCO1B1/1A2	rs4149057	12	21222866
SLCO1B1/1A2	rs6487213	12	21224533
SLCO1B1/1A2	rs11045834	12	21232363
SLCO1B1/1A2	rs4149061	12	21241935
SLCO1B1/1A2	rs4149064	12	21242128
SLCO1B1/1A2	rs11045889	12	21280304
SLCO1B1/1A2	rs2199763	12	21280975
SLCO1B1/1A2	rs12371604	12	21282603
CBR3	rs2242801	21	36424820
CBR3	rs2239566	21	36428636
CBR3	rs2835286	21	36440232
CBR3	rs8129031	21	36445883
CBR3	rs726271	21	36447405
CBR3	rs2835288	21	36447699
ABCG1	rs748319	21	42486126
ABCG1	rs8131660	21	42488628
ABCG1	rs1378577	21	42492734
ABCG1	rs1117640	21	42498343
ABCG1	rs221948	21	42506262
ABCG1	rs4148095	21	42509025
ABCG1	rs3787968	21	42523456
ABCG1	rs225440	21	42526122
ABCG1	rs225448	21	42532899
ABCG1	rs4148117	21	42539134
ABCG1	rs12329683	21	42542679
ABCG1	rs9982242	21	42544189
ABCG1	rs225374	21	42544702
ABCG1	rs225376	21	42547191
ABCG1	rs7279750	21	42547753
ABCG1	rs183436	21	42550900
ABCG1	rs3787986	21	42551877
ABCG1	rs225385	21	42555755
ABCG1	rs17767083	21	42557390
ABCG1	rs225391	21	42557737
ABCG1	rs225398	21	42561663
ABCG1	rs691687	21	42562939
ABCG1	rs2298689	21	42569902
ABCG1	rs2234718	21	42570264
ABCG1	rs4148123	21	42571320
ABCG1	rs182694	21	42571884
ABCG1	rs3787997	21	42572165
ABCG1	rs225406	21	42572299
ABCG1	rs2839482	21	42573501
ABCG1	rs225410	21	42573976
ABCG1	rs3788007	21	42579845
ABCG1	rs450808	21	42580013
ABCG1	rs425215	21	42580170
ABCG1	rs7277991	21	42582751
ABCG1	rs4148137	21	42583121
ABCG1	rs914189	21	42583978
ABCG1	rs9975333	21	42587586

SLCO1B1	rs55737008	12	21283314
SLCO1B1/1A2	rs2199680	12	21306763
SLCO1B1/1A2	rs11045913	12	21307680
SLCO1B1/1A2	rs11045945	12	21340335
SLCO1B1/1A2	rs16923647	12	21342662
SLCO1B1/1A2	rs3751270	12	21348518
SLCO1B1/1A2	rs4115170	12	21366230
SLCO1B1/1A2	rs7958005	12	21369104
SLCO1B1/1A2	rs4762818	12	21384796
SLCO1B1/1A2	rs11837182	12	21393223
SLCO1B1/1A2	rs4762699	12	21395335
SLCO1B1/1A2	rs17333533	12	21396583
SLCO1B1/1A2	rs10743413	12	21398341
SLCO1B1/1A2	rs11045994	12	21398969
SLCO1B1/1A2	rs10841798	12	21403464
SLCO1B1/1A2	rs2045938	12	21411619
SLCO1B1/1A2	rs7137767	12	21416873
SLCO1B1/1A2	rs5486	12	21423367
SLCO1B1/1A2	rs2417977	12	21424435
SLCO1B1/1A2	rs7967902	12	21434482
SLCO1B1/1A2	rs11046012	12	21436623
ABCC9	rs4148679	12	21857968
ABCC9	rs4148677	12	21867695
ABCC9	rs2112080	12	21874187
ABCC9	rs1517284	12	21879355
ABCC9	rs704175	12	21882887
ABCC9	rs704179	12	21884887
ABCC9	rs829080	12	21891130
ABCC9	rs1283822	12	21893150
ABCC9	rs4148674	12	21894811
ABCC9	rs704189	12	21903614
ABCC9	rs697252	12	21909431
ABCC9	rs704205	12	21921432
ABCC9	rs4148666	12	21923258
ABCC9	rs7301876	12	21925887
ABCC9	rs4148665	12	21926514
ABCC9	rs1914361	12	21937120
ABCC9	rs4148649	12	21961498
ABCC9	rs2277404	12	21969823
ABCC9	rs10841914	12	21986068
ALDH2	rs737280	12	110679359
ALDH2	rs2238151	12	110696216
ALDH2	rs968529	12	110718751
ABCG1	rs1541290	21	42591552
ABCG1	rs3746917	21	42591941
ABCG1	rs15661	21	42597422
ABCG1	rs225434	21	42597810
SLC19A1	rs3753019	21	45749213
SLC19A1	rs2236483	21	45750482
SLC19A1	rs2838950	21	45750725
SLC19A1	rs7278425	21	45750979
SLC19A1	rs2838951	21	45754148
SLC19A1	rs17004785	21	45757046
SLC19A1	rs2838956	21	45769452
SLC19A1	rs3788205	21	45788806
GSTT2	rs575959	22	22619365
GSTT2	rs113413	22	22622264
GSTT2	rs2858908	22	22625776
CYB5R3	rs137043	22	41295611
CYB5R3	rs137049	22	41298166
CYB5R3	rs1540311	22	41337538
CYB5R3	rs137111	22	41338048
CYB5R3	rs137116	22	41341618
CYB5R3	rs137124	22	41345660
CYB5R3	rs743887	22	41348531
CYB5R3	rs137126	22	41349196
CYB5R3	rs137127	22	41349576
CYB5R3	rs2071846	22	41353883
CYB5R3	rs137138	22	41353924
CYB5R3	rs137139	22	41354354
CYB5R3	rs2267452	22	41355414
CYB5R3	rs737733	22	41356240
CYB5R3	rs2071847	22	41357574
CYB5R3	rs6002829	22	41357768
CYB5R3	rs3091344	22	41358425
CYB5R3	rs5751318	22	41365737
CYB5R3	rs6002850	22	41371489
CYB5R3	rs9611830	22	41382080
CYB5R3	rs1009433	22	41383757
	rs2106806	X	15557622
	rs2227144	X	18018148
	rs1927286	X	55300517
	rs1386582	X	63901233
	rs10217898	X	63965932
	rs1932206	X	64132368
	rs10126240	X	64176284

ALDH2	rs4646778	12	110720166
ALDH2	rs16941667	12	110728796
ALDH2	rs16941669	12	110730020
ABCC4	rs7321486	13	94462890
ABCC4	rs4148551	13	94471519
ABCC4	rs4274307	13	94478310
ABCC4	rs6492763	13	94482701
ABCC4	rs17189299	13	94483795
ABCC4	rs2182262	13	94489513
ABCC4	rs1151471	13	94495881
ABCC4	rs1189446	13	94505114
ABCC4	rs9561773	13	94505835
ABCC4	rs1750190	13	94507073
ABCC4	rs12854072	13	94507444
ABCC4	rs1189457	13	94517495
ABCC4	rs1189465	13	94524074
ABCC4	rs1751045	13	94528093
ABCC4	rs943290	13	94529059
ABCC4	rs1751052	13	94531379
ABCC4	rs12864844	13	94533730
ABCC4	rs4148530	13	94534997
ABCC4	rs1751059	13	94554024
ABCC4	rs1729775	13	94555944
ABCC4	rs4773840	13	94568426
ABCC4	rs1189428	13	94578020
ABCC4	rs1729741	13	94582157
ABCC4	rs2766482	13	94583722
ABCC4	rs4148514	13	94587147
ABCC4	rs4148512	13	94589729
ABCC4	rs3852616	13	94591375
ABCC4	rs9590190	13	94600527
ABCC4	rs1729788	13	94606004
ABCC4	rs1750996	13	94609600
ABCC4	rs1038138	13	94615397
ABCC4	rs9561797	13	94618853
ABCC4	rs11568663	13	94620762
ABCC4	rs1729786	13	94621240
ABCC4	rs17189376	13	94622104
ABCC4	rs1887162	13	94633634
ABCC4	rs3843689	13	94636242
ABCC4	rs1557069	13	94637297
ABCC4	rs4773843	13	94637496
ABCC4	rs1611822	13	94640070
	rs6625400	X	68270595
ABCB7	rs5937937	X	74185882
	rs1023465	X	78104115
	rs2499043	X	118257334
	rs1541341	X	118893264
	rs6637326	X	126819324

ABCC4	rs9524821	13	94641435
ABCC4	rs2487566	13	94643273
ABCC4	rs4148482	13	94643632
ABCC4	rs1678384	13	94646394
ABCC4	rs1678386	13	94647177
ABCC4	rs1751022	13	94647911
ABCC4	rs17268163	13	94651236
ABCC4	rs1678388	13	94651781
ABCC4	rs1751027	13	94651871
ABCC4	rs4773844	13	94653501
ABCC4	rs1617844	13	94656136
ABCC4	rs2274405	13	94656979
ABCC4	rs873706	13	94660388
ABCC4	rs1926657	13	94672957
ABCC4	rs9561811	13	94681672
ABCC4	rs4148455	13	94686278
ABCC4	rs9634642	13	94686672
ABCC4	rs4148454	13	94687506
ABCC4	rs9524858	13	94690357
ABCC4	rs4148450	13	94693667
ABCC4	rs7320375	13	94709934
SLC15A1	rs4646214	13	98182341
SLC10A2	rs9586056	13	102511975
SLC10A2	rs9557997	13	102514832
CYP1A1	rs1048943	15	72800038
SLCO3A1	rs9989284	15	90414958
ABCC1/6	rs903880	16	16038015
SULT1A1	rs9282861	16	28525015
ABCC3	rs739921	17	46074589
CYP4F11	rs17641483	19	15919371
CYP2A6	rs28399468	19	46041572
CYP2A6	rs5031016	19	46041614
CYP2A13/2F1	rs1645674	19	46334111
CYP24A1	rs3787555	20	52216098
SLC19A1	rs12483553	21	45750521

Appendix4: Primers and Oligos for Spike in Assays.

Table1: PCR Primers for Spike in Products

Name	F Primer	R Primer	Amp Size	Chr	Start	End
2D6_1	CACTGGCTCCAAGCATGGCAG	CCGGATTCCAGCTGGGAAATG	3167	22	40855423	40858590
2D6_2	CTGGAATCCGGTGTCGAAGTGG	CGGCCCTGACACTCCTTCTTG	1807	22	40853626	40855433
2D6_deletion	GCTAATTGGTGCATGCACGAACC	GCATGAGCTAAGGCACCCAGAC	3650	22	na	na
2D6_duplication	CACCATGGTGTCTTTGCTTTCCTGG	AGCACCTCAGGTCAGTCACG	3900	22	na	na
2C19_1	ATGTCTGGAGGAGACCAGGA	CGAAGATTAGGAGACTTTGTCCTT	678	10	96511316	96511994
2C19_2	GCTTCAACCTAGTACAATGAAACCA	AATCTCTAATAACAAACACTTCCCTTACTG	752	10	96512205	96512957
2C19_3	CTGTATTTTGGCCTGGAACG	TCTCAGCTTCAAACCCTGCT	594	10	96524835	96525429
2C19_4	CAGCTAGGCTGTAATTGTTAATTCG	AGGGCTTTGGAGTTTAGTGGA	619	10	96530044	96530663
2C19_5	GGCTTGTCCTTCAATTCACAC	TTTTACATTTTCTATGATGCTTACTGG	1160	10	96530755	96531915
2C19_6	AGATCTGAAGATAGGTGAAGAGTAAGC	TCAATGTAACTATTATAGAGTATGGGGAAT	643	10	96602225	96602868
2C9_1	ATGGGGAGGATGGAAAACAGAGACTT	AGTAGAGAAGATAGTAGTCCAGTAAGGTCAGTGATATG	335	10	96691807	96692142
2C9_2	AGAATCAATGGACATGAACAACCCTCA	AGTTAAACATCCTTAGTAAACACAGAACTAGTCAACA	151	10	96698968	96699119
2C9_3	CTAAAGTCCAGGAAGAGATTGAACGTGTG	GGAGAAACAAACTTACCTTGGGAATGAGA	203	10	96730930	96731133

Table1 contains PCR primers to amplify specific regions in CYP2C9, CYP2D6 and CYP2C19. The 2D6_deltion and 2D6_duplication primers only produce a PCR product when either the deletion or duplication is present respectively. The PCR product can then be spiked into the GoldenGate process in place of genomic DNA.

Table2: Custom GoldenGate Oligos Designed for the PCR ProductVariants

Haplotype	Gene	Chr	Position	Variation	Oligos
***7**	CYP2D6	22	40853802	T/G	ACTTCGTCAGTAACGGACCTCACGCTGCACATCCGGAT GAGTCGAGGTCATATCGTCTCACGCTGCACATCCGGAG TAGGATCATGAGCAGGAGGTGGACGGTGCTGAACATCCAGTGTCTGCCTATAGTGAGTC
***5**	CYP2C19	10	96602485	A/G	ACTTCGTCAGTAACGGACGCCCTCTCCCACACAAATCCA GAGTCGAGGTCATATCGTGCCCTCTCCCACACAAATCCG TTTCCTGAAAATAACAAACATAGGGCCCCATTCGATGTACCTGAGAGTCTGCCTATAGTGAGTC
***4**	CYP2C9	10	96731044	A/G	ACTTCGTCAGTAACGGACGGCTGGTGGGGAGAAGGTCAA GAGTCGAGGTCATATCGTGGCTGGTGGGGAGAAGGTCAG GTATCTCTGGACCTCGTGCCGAGCGGCAGTTAGTCATGTCGGTCTGCCTATAGTGAGTC
***14**	CYP2D6	22	40854979	A/G	ACTTCGTCAGTAACGGACGGCCTTCGCCAACCACTCCA GAGTCGAGGTCATATCGTGGCCTTCGCCAACCACTCCG TGGGTGATGGGCAGAAGAGACCTTCGGCTCTTATGCCCAGTCTGCCTATAGTGAGTC
***12**	CYP2D6	22	40856614	T/C	ACTTCGTCAGTAACGGACCAGCAGGTTGCCCAGCCT GAGTCGAGGTCATATCGTCAGCAGGTTGCCCAGCCC CTGGTGGGTAGCGTGCAGGGCGGCCTATCAAATCACTGGCGTCTGCCTATAGTGAGTC
***11**	CYP2D6	22	40855856	G/C	ACTTCGTCAGTAACGGACCCCTGACCCTCCCTCTGCAG GAGTCGAGGTCATATCGTCCCTGACCCTCCCTCTGCAC CTTCGGGGACGTGTTCAGGAATTTAGCCCGCATCCTACCTGGTCTGCCTATAGTGAGTC
***8**	CYP2D6	22	40854979	T/G	ACTTCGTCAGTAACGGACGGCCTTCGCCAACCACTCCT GAGTCGAGGTCATATCGTGGCCTTCGCCAACCACTCCG TGGGTGATGGGCAGAAGCCTGTCCGAAGTGTGTGCCAAAGTCTGCCTATAGTGAGTC

***3**	CYP2D6	22	40854188	T/G	ACTTCGTCAGTAACGGACGCTGGGTCCCAGGTCATCCT GAGTCGAGGTCATATCGTGCTGGGTCCCAGGTCATCCG TCAGTTAGCAGCTCATCCAGTGAATCCCCTACATACTGCGCGGTCTGCCTATAGTGAGTC
***12**	CYP2C19	10	96602661	T/G	ACTTCGTCAGTAACGGACCAGCCAGACCATCTGTGCTTCTT GAGTCGAGGTCATATCGTCAGCCAGACCATCTGTGCTTCTG AGACAGGAATGAAGCACAGCCGCAGACTGGAGGACTCGGTATGTCTGCCTATAGTGAGTC
***9**	CYP2D6	22	40848676	A/G	ACTTCGTCAGTAACGGACGCCTTCCTGGCAGAGATGGAGA GAGTCGAGGTCATATCGTGCCTTCCTGGCAGAGATGGAGG GAGTGGCTGCCACGGTTCTATGAGGGCGAAGGAGCCTAGTCTGCCTATAGTGAGTC
***17**	CYP2C19	10	96511647	T/C	ACTTCGTCAGTAACGGACGTCAAATTTGTGTCTTCTGTTCTCAAAGT GAGTCGAGGTCATATCGTGTCAAATTTGTGTCTTCTGTTCTCAAAGC TCTCTGATGTAAGAGATAATGCGAGCGTACTCGACCGATCCCTGGGTCTGCCTATAGTGAGTC
***4**	CYP2C19	10	96512453	T/C	ACTTCGTCAGTAACGGACCACAAGGACCACAAAAGGATCCAT GAGTCGAGGTCATATCGTCACAAGGACCACAAAAGGATCCAC GAAGCCTTCTCCTCTTGTTAAGTCCCAGCGGAGATAATCCCGTCGTCTGCCTATAGTGAGTC
***17**	CYP2C19	10	96592613	T/C	ACTTCGTCAGTAACGGACGGCTCCGGTTTCTGCCAAT GAGTCGAGGTCATATCGTGGCTCCGGTTTCTGCCAAC ACACGTTCAATCTCTTCCTGACGACTTCGTATAGGCTGGCAGGTCTGCCTATAGTGAGTC
***8**	CYP2C19	10	96525163	A/G	ACTTCGTCAGTAACGGACAGAAACGCCGGATCTCCTTCCA GAGTCGAGGTCATATCGTAGAAACGCCGGATCTCCTTCCG CTCTTTCCATTGCTGAAAACGTTCCCGCAACAGGAGGGATTCGTCTGCCTATAGTGAGTC
***2**	CYP2C19	10	96531606	T/C	ACTTCGTCAGTAACGGACGGTTTTTAAGTAATTTGTTATGGGTTCCT GAGTCGAGGTCATATCGTGGTTTTTAAGTAATTTGTTATGGGTTCCC GGAAATAATCAATGATAGTGGGAACAAGCTCCACAGTCATCCGGTGTCTGCCTATAGTGAGTC
***3**	CYP2C19	10	96530400	A/G	ACTTCGTCAGTAACGGACTCAGGATTGTAAGCACCCCCTGA GAGTCGAGGTCATATCGTTCAGGATTGTAAGCACCCCCTGG TCCAGGTAAGGCCAAGTTTTGGCGCTTTCAAGAGGCGTAACTGTCTGCCTATAGTGAGTC
***3**	CYP2C9	10	96731043	T/G	ACTTCGTCAGTAACGGACGCTGGTGGGGAGAAGGTCAAT GAGTCGAGGTCATATCGTGCTGGTGGGGAGAAGGTCAAG CACCACAGCATCTGTGTAGGGTGCCTCCATACAAACGCTGGGGTCTGCCTATAGTGAGTC

***2**	CYP2C9	10	96692037	T/C	ACTTCGTCAGTAACGGACGGGGGAAGAGGAGCATTGAGGACT GAGTCGAGGTCATATCGTGGGGGAAGAGGAGCATTGAGGACC CCTTGTGGAGGAGTTGAGAAGTATTTACCCCTGAGCCGGTCAGTCTGCCTATAGTGAGTC
***9**	CYP2C9	10	96698964	T/C	ACTTCGTCAGTAACGGACTTGTTCATGTCCATTGATTCTTGGT GAGTCGAGGTCATATCGTTTGTTCATGTCCATTGATTCTTGGC TTCTTTTACTTTTTCCAAAATATAATGAGGTGACGCATCCCTCCATTGTCTGCCTATAGTGAGTC
***11**	CYP2C9	10	96730971	A/G	ACTTCGTCAGTAACGGACGGTCTTGCATGCAGGGGCTCCA GAGTCGAGGTCATATCGTGGTCTTGCATGCAGGGGCTCCG TTTCTGCCAATCACACGTTGAAGTCAGGTCAATCGTCGCGCGTCTGCCTATAGTGAGTC
***5**	CYP2C9	10	96731048	G/C	ACTTCGTCAGTAACGGACGCAGGCTGGTGGGGAGAAGG GAGTCGAGGTCATATCGTGCAGGCTGGTGGGGAGAAGC CAATGTATCTCTGGACCTCGTAGAAATTACGGTCCTCCACCGACGTCTGCCTATAGTGAGTC
***8**	CYP2C9	10	96692056	T/C	ACTTCGTCAGTAACGGACGTTCTCAACTCCTCCACAAGGCAGT GAGTCGAGGTCATATCGTGTTCTCAACTCCTCCACAAGGCAGC GGCTTCCTCTTGAACACGGGATGCTGGGAGACGTTCACATGTCTGCCTATAGTGAGTC
***10**	CYP2C9	10	96699027	A/G	ACTTCGTCAGTAACGGACTTGATTGCTTCCTGATGAAAATGGA GAGTCGAGGTCATATCGTTTGATTGCTTCCTGATGAAAATGGG AAGGTAAAATGTAAACAAAAGCTTTCGATACCGCAAGGTCATTGGCGTCTGCCTATAGTGAGTC
***12**	CYP2C9	10	96738767	A/G	ACTTCGTCAGTAACGGACGGCCATCTGCTCTTCTTCAGACAGA GAGTCGAGGTCATATCGTGGCCATCTGCTCTTCTTCAGACAGG ATGAAGCACAGCTGGTAGAAGATCGTCCCATCGCAAGCCTGAGGTCTGCCTATAGTGAGTC
***10**	CYP2D6	22	40856638	A/G	ACTTCGTCAGTAACGGACGAGTGGCAGGGGGCCTGGTGA GAGTCGAGGTCATATCGTGAGTGGCAGGGGGCCTGGTGG TAGCGTGCAGCCCAGCTCCCCAGTAGAGTACGGCGGTCGTCTGCCTATAGTGAGTC
***2a**	CYP2D6	22	40858326	C/G	ACTTCGTCAGTAACGGACGCCTGGACAACTTGGAAGAACCC GAGTCGAGGTCATATCGTGCCTGGACAACTTGGAAGAACCG AAATTAGCTGGGATTGGGTCGTAAGCTCGACTGTAGACGATGGTCTGCCTATAGTGAGTC
***2**	CYP2D6	22	40852557	G/C	ACTTCGTCAGTAACGGACGACAAAGCTCATAGGGGGATGGGG GAGTCGAGGTCATATCGTGACAAAGCTCATAGGGGGATGGGC CACCAGGAAAGCAAAGACACGGTAGTCGTTCACAGGGCAAGCGTCTGCCTATAGTGAGTC

***17**	CYP2D6	22	40855716	T/C	ACTTCGTCAGTAACGGACCCCGCCTGTGCCCATCAT GAGTCGAGGTCATATCGTCCCGCCTGTGCCCATCAC CAGATCCTGGGTTTCGGAGAATTACCCGCTCGCTACGGAGTCTGCCTATAGTGAGTC
***41**	CYP2D6	22	40853749	T/C	ACTTCGTCAGTAACGGACGCCCCCGCCTGTACCCTTT GAGTCGAGGTCATATCGTGCCCCCGCCTGTACCCTTC CTGCACTGTTTCCCAGATGACATAGGTGCGGCTGGGCTTACGTCTGCCTATAGTGAGTC
***4**	CYP2D6	22	40854891	A/G	ACTTCGTCAGTAACGGACGCGCATCTCCCACCCCCAA GAGTCGAGGTCATATCGTGCGCATCTCCCACCCCCAG CAACGGTCTCTTGGACAAAGGGGTTAGGTGATACACCGAGCTGTCTGCCTATAGTGAGTC
***5**	CYP2D6				ACTTCGTCAGTAACGGACTTGGTGCATATACAATCCTCTG ACTTCGTCAGTAACGGACTTGGTGCATATACAATCCTCTG GTGGGGACTTGGAGAACTTTACTCTAAGGCGGGTCTGCTCGAGTCTGCCTATAGTGAGTC
XN	CYP2D6				ACTTCGTCAGTAACGGACCCAGGCACCTAAGGAACTCT ACTTCGTCAGTAACGGACCCAGGCACCTAAGGAACTCT ATTAGCTGACCACTGCCGTACGATCTCTTGGTCGCAAGTATCCGTCTGCCTATAGTGAGTC

Table2 contains the custom designed GoldenGate oligos to assay variants in the PCR amplicons for CYP2D6, CYP2C9 and CYP2C19. The three oligos in order are the two allele specific oligos and the locus specific oligo. For the CYP2D6 deletion (*5) and the CYP2D6 duplication (XN), signal is generated if the deletion or duplication is present respectively.

Appendix 5: Additional Phenotypes in IKP liver samples

The following two tables contain enzymatic activity and protein expression data for selected ADME enzymes. Each phenotype was tested for association with each SNP on the ADME panel. Each phenotype was treated as both a continuous and dichotomous trait.

Table 1: Enzymatic Activities (Enzyme name and probe drug measured)

Sample	1A2 Phenacetin	2A6 Coumarin	2B6 OH-Bup	2C8 Amodiaquine	2C9 LucH	2C19 4OH-S-Meph	2D6 5OH-PPF-5	2E1 Chlorzoxazone	3A4 oOH-ATV	POR cycOx
16	1353.05	879.33	81.73	1722.83	40.26	179.24	91.39	86.36	193.21	0.12
18	1211.76	848.69		1448.47	50.73	40.09	67.62	99.50	110.79	0.14
19	1988.03	1424.92		965.81	88.85	39.27	20.40	196.93	188.81	0.15
21	1092.03	682.30	49.47	1333.47	14.96	13.53	45.89	362.83	128.35	0.12
22	1618.64	573.17	74.10	1972.39	64.39	59.03	68.23	186.18	430.54	0.23
25	1315.44	96.80	47.47	1442.71	27.28	51.26		67.99	543.58	0.18
28	1312.56	2081.04	218.73	2115.54	27.39	67.63	45.71	75.23	275.30	0.13
29	2390.98	1050.06	######	2578.11	24.65	27.85	56.52	310.74	447.50	0.18
36	1138.77	1366.55	107.87	1762.00	23.20	69.61	111.89	109.79	216.16	0.16
38	1517.34	1642.33	118.73	1788.39	22.16	171.63	95.63	175.36	346.74	0.13
39	2902.26	2244.92	137.73	3117.21	43.80	188.06	86.55	110.06	283.21	0.20
40	1612.33	1658.45	74.06	1182.79	22.68	45.39	109.87	76.68	460.64	0.15
41	2354.26	1405.47	163.13	1034.97	31.93	78.93	39.59	196.30	484.30	0.18
42	2752.73	1356.23	158.33	1503.19	24.77	43.61	78.58	83.29	196.08	0.15
43	1381.62	1751.77	131.33	1034.86	10.35	52.12	166.95	87.57	310.31	0.15
44	616.01	1673.39	39.33	1276.35	5.09	11.93	46.13	53.95	116.91	0.13
47	250.92	300.70	232.46	756.53	22.28	11.95	41.02	79.72	4.99	0.12
54	813.42	1601.73	194.13	1158.87	20.75	51.25	175.41	113.83	224.24	0.16
58	3069.27	1333.97	161.80	2666.28	20.00	65.65	143.75	114.70	410.75	0.19

59	2101.52	598.04	290.66	1857.96	31.41	90.11	59.85	69.70	292.91	0.14
61	1589.13	1088.41	122.73	1325.51	38.95	50.78	31.11	69.16	166.30	0.11
63	1642.44	771.89	65.73	1352.33	40.61	53.40	111.46	93.67	215.09	0.17
64	701.49	1115.30	105.47	1316.53	24.96	57.08	91.93	54.46	140.01	0.13
69	1123.23	1746.74	331.47	1251.53	29.72	12.19	63.27	180.85	484.15	0.15
70	2526.26	639.03	70.80	958.41	17.15	18.07	0.07	93.44	169.57	0.09
72	664.50	779.09	280.67	659.31	30.20	1.58	128.12	38.52	115.70	0.20
76	1513.27	1113.03	32.27	1284.42	13.03	9.56		86.55	263.12	0.12
79	460.51	142.52	93.40	426.68	16.67	1.24	5.28	52.50	53.49	0.11
80	2193.28	1612.83	102.00	2007.76	42.99	94.26	12.72	149.57	519.43	0.16
81	2014.08	867.93	143.49	1553.63	64.69	64.39	182.96	183.44	242.50	0.22
82	2355.42	383.70	119.60	847.90	58.11	60.00	56.18	123.87	262.40	0.16
85	598.17	1088.23	67.93	694.60	27.36	35.01		65.45	169.85	0.10
87	1919.99	841.68	76.40	1744.91	59.77	1.58		97.70	188.76	0.14
96	1435.50	1081.36	154.93	1547.65	39.63	125.68		98.23	341.37	0.13
98	3383.42	1167.49	74.00	1470.88	29.50	87.65	35.14	71.30	121.97	0.10
102	3423.65	2360.95	197.00	2018.22	30.36	37.56		138.45	416.03	0.19
103	1269.51	525.94	193.33	1620.43	43.75	24.37	56.65	121.33	88.00	0.12
104	981.51	639.50	114.80	1278.64	74.05	55.04	0.00	56.84	251.75	0.12
106	1446.92	379.13	45.30	973.83	79.38	41.85	63.60	112.23	349.59	0.15
118	2751.06	1999.95	273.00	2233.55	53.98	79.33	17.48	69.54	192.84	0.14
120	1338.91	1176.56	29.47	1874.92	10.84	52.27	75.15	64.34	140.60	0.11
121	4422.95	1967.03	98.27	427.81	43.50	118.58		179.77	263.98	0.16
122	2352.98	2657.17	285.00	2436.65	73.21	49.19		258.80	358.52	0.17
123	194.85	298.11	49.53	2137.41	9.10	1.07		82.37	60.50	0.12
124	4447.98	1245.11	94.87	1031.02	45.08	24.97		155.67	169.83	0.19
126	1951.60	1545.58	79.67	1896.28	76.71	107.21	171.42	84.38	137.86	0.14

127	825.30	374.14	19.60	1281.53	45.67	2.42	109.58	175.80	208.39	0.10
129	2871.86	1965.47	57.00	2287.46	51.58	93.63	258.03	519.90	248.06	0.17
132	1986.58	996.79	98.48	1382.51	45.60	1.53	32.48	158.52	63.86	0.12
133	4536.21	680.98	129.63	1843.28	74.37	66.27	87.06	157.60	267.89	0.18
134	4022.85	2516.59	139.08	1773.66	91.16	80.22	135.25	301.39	401.26	0.14
135	1219.50	1501.34	71.01	1048.84	33.26	186.76	344.50	166.44	147.32	0.11
136	1554.11	2887.51	335.62	2428.11	147.53	115.65	49.76	242.26	286.77	0.22
137	194.85	310.74	129.81	294.88	16.63	0.76	99.38	127.85	8.88	0.19
139	1169.32	631.21	79.22	1414.91	16.91	112.55	110.13	578.85	279.17	0.16
140	2823.66	2010.07	211.06	1680.26	37.55	120.44	157.07	306.10	454.83	0.14
143	2580.92	2118.18	265.24	1902.42	42.38	180.80	66.84	286.81	249.71	0.21
144	875.06	1927.53	199.35	1705.11	20.97	106.07	115.05	166.63	87.69	0.10
145	1919.91	687.56	75.24	912.30	15.26	6.99	123.45	196.91	195.18	0.13
150	1044.93	1452.49	7.10	850.66	17.43	102.89	114.14	286.90	164.17	0.14
153	3488.63	639.95	5.95	771.43	20.78	25.74	154.82	358.34	271.30	0.18
156	852.12	1409.23	51.91	700.57	29.39	48.79	71.42	173.04	177.86	0.14
157	1852.14	2276.89	5.56	1130.98	59.09	138.24	66.86	269.92	264.06	0.15
159	579.39	663.85	76.72	710.41	5.31	59.60	106.78	383.07	103.73	0.11
163	2348.01	940.91	8.95	1023.53	25.00	108.51	4.26	396.29	333.46	0.21
164	1141.04	881.10	87.40	1323.71	14.95	40.89	148.21	203.77	237.88	0.12
165	2668.28	2811.31	9.40	1800.82	30.33	60.40	24.97	310.70	287.60	0.12
166	1993.35	1347.39	147.61	1428.39	20.88	38.05	186.79	404.59	163.62	0.14
168	1365.05	3624.26	######	2632.33	47.72	159.45	38.85	513.34	1397.27	0.26
169	130.12	450.91	123.74	326.27	8.86	0.85	66.03	79.74	23.10	0.14
170	1476.75	1615.53	92.70	1316.78	41.71	23.77	187.09	166.56	156.67	0.15
171	4071.72	1282.09	41.36	960.23	22.76	91.79	125.55	340.79	173.18	0.18
178	197.32	416.15	31.73	444.79	16.96	0.63	87.03	86.72	24.91	0.14

179	2382.71	574.95	38.46	847.68	44.69	10.49	370.69	442.18	271.81	0.20
181	1079.21	771.58	97.19	927.62	21.94	6.61	47.86	146.25	146.32	0.12
182	751.48	1130.89	281.97	944.90	37.24	12.41	53.47	165.94	231.08	0.14
183	1610.88	736.77	23.61	860.85	24.62	17.06	13.58	150.73	122.59	0.11
186	390.98	1386.59	37.72	616.57	11.91	2.08	72.17	118.47	34.07	0.09
187	2650.17	1309.10	83.59	861.14	20.17	2.51	433.00	422.57	134.47	0.18
188	1555.72	1258.90	35.56	1086.43	15.60	22.23	48.72	266.25	153.72	0.14
189	3383.39	1172.69	193.22	1188.58	12.91	39.49	121.36	242.17	314.78	0.13
190	1702.59	1649.02	95.15	760.23	11.61	151.32	129.12	227.87	387.69	0.15
191	707.42	1411.72	616.92	1307.24	22.36	63.70	54.65	330.12	572.73	0.21
192	1210.21	679.16	56.74	1285.66	16.61	49.54	50.75	122.79	82.49	0.13
193	1437.13	558.33	137.80	1474.70	12.03	118.79	67.41	402.17	243.61	0.12
194	1374.47	2609.83	249.61	1402.22	20.35	45.68	170.60	491.22	266.20	0.13
195	2262.50	653.45	29.30	869.63	16.02	16.46	5.50	192.72	159.32	0.08
199	1157.70	1060.03	61.56	1082.79	20.19	150.25	61.77	327.23	86.35	0.11
202	998.26	761.52	79.97	504.92	18.46	1.19	168.84	187.15	57.42	0.20
205	1082.55	1234.09	89.03	1120.00	19.86	1.79	79.33	140.82	66.18	0.11
209	1974.15	1780.73	163.78	1098.57	26.66	8.68	88.09	209.55	342.34	0.11
210	1316.11	638.96	63.62	693.90	11.28	39.36	167.64	142.39	182.56	0.12
212	1977.90	799.68	59.45	442.80	27.58	58.47	55.19	62.67	115.00	0.10
213	1487.63	202.09	96.69	603.20	19.28	32.89	108.21	321.85	44.92	0.11
214	442.32	849.13	83.42	850.87	16.45	2.41	97.68	135.37	85.75	0.08
218	1957.19	1642.52	152.23	1199.14	22.00	19.36	1.90	258.17	0.31	0.13
221	716.32	449.63	73.10	1276.12		8.18	94.10	272.50	55.88	0.09
222	784.77	760.56	39.16	902.47	21.19	48.35	81.31	210.18	127.84	0.10
223	1842.98	2144.77	160.50	1151.56	14.46	57.58	209.16	441.17	192.35	0.13
224	2024.86	939.00	125.60	1153.95	18.96	70.48	108.76	484.84	71.00	0.10

225	2535.70	2076.01	116.34	776.49	13.22	22.52	56.46	609.20	303.49	0.19
226	1013.93	1033.19	91.86	911.02		43.11	82.76	115.07	190.19	0.20
227	1345.76	1381.38	79.32	927.14		81.20	4.69	269.61	122.26	0.11
229	1810.89	1438.89	54.54	1863.82	36.27	133.09	106.47	376.89	306.60	0.15
230	1500.86	893.00	58.00	1041.85	15.15	43.78	118.52	94.60	131.09	0.10
231	1537.38	819.06	143.35	1413.37	22.56	11.49	254.82	329.51	73.71	0.17
232	1635.49	468.07	62.56	995.64	15.82	11.44	247.44	860.36	33.37	0.21
234	562.53	166.00	65.43	734.23	16.21	8.88	129.89	190.45	104.60	0.15
235	965.14	2214.72	56.18	1062.24	31.70	4.10	166.62	248.96	240.28	0.22
236	2566.76	2062.08	116.62	1226.01	24.09	153.39	168.78	454.32	397.41	0.19
237	2486.96	1552.35	66.67	1476.83	41.74	9.72	99.26	510.92	176.46	0.19
238	1183.16	1776.29	101.71	1292.91	22.47	11.34	84.37	137.76	448.01	0.13
239	2549.68	623.54	52.75	1186.91	26.91	117.86	99.28	86.33	187.69	0.15
240	2209.16	1880.78	179.93	1680.19	36.94	84.43	101.29	226.16	373.55	0.19
242	1612.07	857.99	50.46	1150.03	13.04	106.11	54.61	118.49	103.98	0.09
243	1464.46	827.81	92.70	1470.46	27.20	31.64	125.48	160.21	180.88	0.13
244	1144.77	1267.00	175.13	1402.79	36.54	273.92	77.02	174.34	430.44	0.15
246	2073.81	1875.58	124.41	1023.18	21.52	67.16	164.66	291.26	243.94	0.16
248	1238.43	580.77	69.80	1182.61	26.31	60.15	152.44	211.48	213.24	0.15
249	918.35	527.05	138.35	1098.77	12.32	44.88		188.89	89.70	0.12
250	1281.31	635.10	45.66	918.39	7.14	14.20	55.38	147.42	130.12	0.14
251	973.89	634.62	148.60	1114.44	26.25	65.24	160.53	123.62	131.51	0.15
254	1250.92	1238.17	18.96	1280.76	17.02	58.37	49.81	160.95	248.49	0.15
255	671.37	643.46	82.71	970.54	23.07	52.04		177.85	109.81	0.09
259	1017.83	1778.66	168.76	1512.00	15.99	46.56		222.24	134.61	0.13
260	677.91	570.12	110.77	603.05	21.57	3.96	121.01	146.47	139.69	0.11
261	1556.73	1372.83	175.25	1158.13	17.91	56.77	123.36	129.77	329.99	0.14

264	718.06	758.45	84.19	722.90	16.93	13.32	61.68	121.71	62.01	0.10
266	1781.20	786.76	92.24	1674.87	27.14	116.97	68.20	235.69	184.17	0.14
268	768.80	251.99	43.40	204.23	6.27	2.40	81.49	139.85	171.60	0.13
269	1706.87	780.73	170.45	1838.28	13.22	238.62		214.48	336.40	0.14
270	2232.52	1922.35	211.50	1332.24	7.43	64.58	132.10	184.17	354.11	0.13
271	1460.91	1197.54	88.40	938.57	14.34	53.23	30.43	119.84	63.34	0.09
272	1323.21	727.22	301.13	1332.69	34.01	91.93	78.68	206.38	240.62	0.14
273	1447.72	1217.73	179.30	1618.45	17.24	15.05	152.87	130.15	150.07	0.11
274	878.18	379.12	152.30	831.51	21.91	3.03	1.44	200.53	96.09	0.14
275	1357.18	690.21	106.98	1230.85	44.07	97.57	177.06	185.70	222.71	0.25
279	1554.56	1419.84	86.22	552.79	56.59	126.76	187.20	127.47	203.65	0.18
281	235.45	1629.32	87.41	342.81	25.04	1.97	57.34	69.36	62.71	0.20
282	458.45	1218.33	86.07	786.77	28.65	25.97	126.99	21.51	94.59	0.20
283	284.01	604.45	14.70	645.33	26.20	17.37	53.90	45.69	53.30	0.10
285	961.16	1080.48	39.30	1130.88	18.47	53.37	270.46	103.20	141.16	0.12
288	1588.94	2834.02	671.54	3212.17	56.98	93.43	43.60	121.91	599.18	0.17
293	1232.60	2179.83	866.69	2871.21	31.86	34.31	84.38	178.13	841.09	0.25
295	837.51	1713.79	94.66	1300.11	18.91	26.05	240.65	113.01	83.64	0.15
296	309.09	474.27	34.65	1055.53	44.74	2.80	90.61	67.43	96.31	0.18
297	1426.83	926.68	72.66	1548.36	36.65	33.09	56.62	106.66	104.49	0.12
298	1018.17	654.69	88.53	1307.97	37.80	49.01	77.87	114.55	208.36	0.12
299	2645.70	768.38	139.99	1235.82	20.78	165.56	116.56	203.15	247.60	0.13
300	1314.20	1115.09	96.95	1257.18	15.23	85.90	157.41	114.51	145.15	0.12

Table 2: Protein Expression of Selected ADME Proteins

Sample	1A1	1A2	2A6	2B6	2C8	2C9	2C19	2D6	2E1	3A4	3A5	P450	POR	UGT1A1	UGT1A3
16	0.65	23.00	12.21	11.76	22.46	47.73	61.06	39.00	45.26	47.56	1.53	387.37	15.00	0.7	95.98
18	0.36	52.80	11.17	18.18	78.50	114.83	4.45	39.60	69.26	83.65	0.90	240.96	27.00	3.63	31.88
19	2.39	76.20	16.45	28.31	63.81	160.84	15.45	12.70	82.24	99.46	1.74	314.41	35.40		76.65
21	1.83	76.58	17.19	7.86	50.19	168.79	4.27	57.90	205.92	60.86	2.70	314.38	22.50	0.53	162.41
22	3.81	128.16	12.79	7.24	172.63	66.44	10.41	34.40	149.27	456.95	2.66	328.42	116.50	4.49	23.53
25	0.95	70.86	1.47	4.05	54.75	169.40	7.56	19.85	33.74	233.92	2.18	314.90	139.00	1.96	15.94
28	2.69	73.53	49.05	8.40	283.31	158.56	9.19	23.35	25.91	155.68	3.21	445.26	41.00	0.57	236.79
29	2.69	93.48	26.09	43.85	59.54	83.76	7.78	46.50	169.29	206.65	2.01	543.27	55.50	3.8	42.50
36	3.30	42.93	44.39	16.05	160.00	151.09	14.69	51.00	28.04	121.12	2.56	377.38	44.33	1.35	18.97
38	6.19	135.92	40.85	20.92	67.14	251.11	26.78	31.20	49.88	150.84	2.76	397.29	49.00	2.42	47.81
39	7.89	106.67	42.58	12.65	236.40	91.91	46.08	18.15	71.54	104.99	3.02	440.00	121.50	2.62	69.66
40	3.60	127.16	43.97	33.27	157.04	138.18	18.34	40.40	24.32	370.65	21.41	480.17	58.00	2.17	31.12
41	5.40	81.60	27.13	30.58	95.88	47.15	26.32	14.80	66.18	244.56	16.29	548.57	43.40	1.85	104.73
42	7.78	196.90	45.98	44.26	186.70	80.75	34.50	21.00	44.92	100.26	1.42	370.35	50.00	2.84	201.12
43	3.73	95.63	45.56	32.39	158.11	50.69	35.71	54.00	70.38	174.34	1.76	345.25	47.00	0.95	40.98
44	0.81	56.51	57.24	6.89	152.09	21.14	8.93	19.70	36.27	53.31	1.14	201.31	24.75	0.28	66.79
47	0.15	8.86	7.08	22.94	23.34	91.01	3.40	14.15	119.53	7.22	0.73	183.95	47.67	2.72	55.40
54	0.47	25.74	22.06	8.00	46.58	103.23	13.23	55.57	94.99	101.29	1.72	339.17	58.50	0.88	153.16

58	4.26	135.43	33.32	12.01	394.61	96.89	50.65	37.00	93.92	179.32	2.43	597.39	74.33	1.19	271.33
59	2.26	143.97	20.55	39.64	119.01	233.93	34.45	18.20	28.87	356.95	0.68	304.15	69.00	0.73	232.18
61	3.85	95.82	22.01	32.14	59.56	216.55	15.12	26.95	13.14	62.50	16.84	311.34	44.33	0.7	216.56
63	1.31	183.66	18.77	9.65	114.30	321.38	5.07	78.05	114.61	139.40	1.59	324.52	84.00	1.81	109.89
64	6.14	50.55	23.93	17.31	71.54	100.82	13.96	14.90	21.95	50.14	1.68	302.07	40.50	0.59	332.13
69	0.58	46.08	37.38	21.80	121.18	78.37	3.40	38.00	167.15	137.64	2.96	355.68	102.00	1.48	48.46
70	8.32	117.54	12.25	11.24	49.52	72.11	9.57	0.50	57.76	116.68	2.10	311.07	31.00	1.69	117.04
72	0.48	20.54	16.55	28.28	50.39	162.51	5.76	34.00	11.19	44.22	1.54	204.84	83.50	2.55	192.73
76	2.12	67.29	21.14	4.06	42.29	24.31	4.37	12.10	49.11	75.11	1.73	290.02	33.00	0.73	38.09
79	0.84	21.75	1.57	12.00	32.98	131.28	4.67	7.40	68.24	13.02	1.32	185.34	18.00	1.29	34.11
80	0.33	157.76	51.26	19.71	226.70	455.36	37.94	13.90	115.57	85.74	1.51	569.69	54.50	0.88	57.91
81	5.13	119.93	16.82	3.83	332.74	281.66	22.70	42.50	72.04	95.76	3.22	338.95	161.00	0.68	485.36
82	2.04	93.36	8.38	17.36	75.07	321.98	6.99	21.60	167.14	123.13	3.68	193.87	43.00	0.92	209.89
85	0.73	21.10	17.14	11.85	140.99	108.38	14.66	36.50	42.26	111.44	22.97	246.67	31.00	0.39	38.09
87	9.90	77.84	18.92	19.05	73.95	192.38	9.83	32.40	62.83	46.02	3.71	456.57	71.67	1.25	65.04
96	2.25	85.00	19.94	19.06	98.05	392.66	36.57	50.00	66.89	133.95	1.54	355.19	39.00	0.63	229.90
98	8.32	126.40	23.53	9.90	43.48	187.88	21.41	48.90	27.27	71.68	2.76	387.64	55.00	0.09	210.52
102	10.97	194.76	56.03	26.90	251.58	81.37	14.90	24.90	70.25	157.19	4.23	634.05	124.00	1.26	88.08
103	3.04	77.09	17.18	15.69	74.09	263.98	5.96	49.70	200.30	71.54	2.14	288.21	63.00	0.18	121.55
104	3.50	73.15	16.35	13.34	93.77	525.63	18.44	4.60	87.10	158.35	2.91	369.44	44.50	0	318.86
106	0.47	31.71	8.16	3.51	93.16	162.87	6.07	36.70	73.31	227.19	1.76	350.57	95.00	0.29	199.64
118	13.66	90.13	34.10	26.37	187.71	175.04	21.21	11.40	28.97	72.55	1.38	476.68	56.00	0.58	57.25
120	2.44	51.72	32.91	1.79	82.80	14.59	21.38	39.10	32.48	61.95	2.30	216.22	33.00	0.13	119.79

121	9.73	176.82	46.67	7.32	110.28	141.94	81.23	7.95	86.48	168.81	3.40	500.64	82.67	0.35	58.13
122	2.74	123.07	58.21	15.49	210.29	220.39	7.10	14.25	98.47	249.44	3.30	611.52	88.50	0.48	53.73
123	0.21	4.94	8.40	1.69	18.61	4.52	4.24	13.00	81.81	15.57	0.59	158.31	59.50	3.93	12.33
124	10.32	81.06	16.70	11.74	259.38	190.68	5.81	14.75	94.01	72.00	3.09	527.59	65.00	0.66	51.09
126	3.88	67.07	25.25	17.77	110.31	156.41	35.60	61.30	37.36	83.58	2.37	454.33	59.50	0.82	59.02
127	0.37	19.76	5.56	3.18	48.29	83.30	1.01	18.60	56.04	60.65	1.64	254.05	43.50	0.29	103.94
129	6.61	54.72	45.46	3.17	132.66	71.10	32.64	59.80	79.54	118.94	2.34	482.61	51.50	1.08	76.63
132	1.77	81.52	19.18	5.10	76.45	119.72	0.10	27.40	29.48	34.65	1.40	268.32	37.00	1.94	95.13
133	6.20	120.05	15.72	10.24	70.00	284.11	14.14	23.00	58.26	81.06	3.00	495.24	18.00	1.31	118.91
134	24.36	207.10	63.44	11.12	136.77	287.22	26.27	69.50	109.69	169.02	1.99	548.59	53.00	1.05	837.67
135	2.63	64.81	39.30	18.36	100.17	82.73	63.51	43.20	46.32	121.18	2.38	334.30	43.33	0.87	116.82
136	1.07	62.24	76.46	61.30	190.63	330.20	16.12	23.80	44.87	295.09	18.20	555.64	87.00	2.7	28.20
137	0.26	7.20	3.19	9.66	33.77	28.68	2.09	35.00	18.13	4.38	1.38	539.87	36.50	0.47	91.04
139	1.49	64.95	30.22	6.01	120.39	298.36	59.98	57.50	316.46	236.18	2.65	292.81	138.50	0.4	175.64
140	6.25	141.73	56.22	19.56	149.74	438.77	48.20	59.90	68.14	120.47	2.82	488.31	42.50	1.14	313.41
143	2.72	173.16	66.06	29.89	141.65	176.15	35.36	35.20	51.30	153.40	2.79	507.25	47.50	1.34	28.20
144	3.32	54.31	23.30	7.60	269.67	72.55	23.20	58.60	47.55	75.12	2.29	211.17	35.33	0.51	93.46
145	2.30	80.10	11.63	2.50	48.40	70.36	2.65	47.50	63.88	101.37	1.03	262.64	39.00	0.34	87.01
150	0.86	39.67	34.89	9.45	130.74	35.06	25.82	44.50	65.49	147.70	1.99	320.58	31.00	0.11	37.95
153	6.36	278.74	14.17	15.93	255.78	173.16	7.84	47.00	170.62	343.18	2.99	491.65	47.67	1.47	136.16
156	1.42	54.83	30.65	6.00	76.29	203.66	12.20	26.70	20.02	109.22	3.29	390.40	36.00	0.65	52.37

157	3.56	118.50	37.04	13.97	143.80	323.11	25.21	17.00	65.87	172.12	2.66	428.42	83.00	4.18	153.08
159	0.71	31.65	11.36	4.19	54.86	87.57	21.82	32.70	178.06	79.19	1.64	310.10	40.50	0.63	43.51
163	4.19	187.66	24.08	25.69	65.55	160.43	38.20	0.65	94.61	313.08	2.49	455.60	78.50	1.33	27.14
164	0.80	104.80	27.75	13.78	88.02	178.92	11.18	30.60	73.37	245.71	2.64	352.94	45.00	0.4	186.11
165	6.35	190.48	73.39	26.56	172.73	96.36	7.95	4.60	62.56	171.42	3.05	500.86	59.50	2.62	369.00
166	6.15	148.17	29.87	6.78	97.46	65.12	10.12	18.90	108.51	124.57	18.94	367.88	43.50	0.93	91.85
168	13.17	56.61	114.37	209.53	492.23	170.92	53.56	25.90	144.56	1034.42	2.03	693.75	96.75	1.86	385.12
169	0.45	3.62	4.22	6.14	39.51	11.97	0.47	14.30	13.74	1.54	2.17	79.29	22.00	0.41	88.59
170	3.25	98.38	31.92	9.94	178.32	158.48	8.41	41.40	31.83	160.83	2.02	329.72	54.50	1.27	173.99
171	5.30	208.16	23.32	2.57	94.46	74.87	18.08	47.90	55.00	232.12	2.41	337.29	73.50	1.23	35.92
178	0.50	7.74	3.99	3.30	31.60	38.10	6.11	27.00	4.26	5.80	1.74	79.00	36.00	0.3	17.56
179	3.39	90.53	9.46	7.59	69.37	156.10	3.35	46.20	115.47	128.06	1.12	294.83	76.67	0.86	86.20
181	3.08	22.98	10.18	9.50	45.55	20.08	3.15	20.60	25.99	69.68	1.35	246.58	11.00	0.03	79.81
182	0.68	23.56	21.92	20.20	71.46	201.66	3.28	17.65	52.56	203.59	2.68	255.71	36.00	2.96	42.30
183	9.57	91.14	20.67	12.10	85.77	181.04	7.57	0.40	33.40	78.03	1.14	251.54	22.00	0.79	39.11
186	0.46	20.55	23.00	3.40	64.40	74.01	2.07	36.15	37.80	36.88	1.26	179.47	20.00	1.32	22.35
187	5.81	145.30	24.53	20.63	74.91	47.93	2.51	41.10	98.65	78.03	1.68	276.40	64.00	1.36	170.80
188	4.40	46.95	25.43	5.01	70.28	76.62	8.78	16.45	50.80	82.69	0.96	211.43	36.00	0.33	170.80
189	2.71	157.05	30.93	19.40	123.66	120.36	13.31	14.90	44.10	207.08	2.92	399.75	46.00	1.23	464.51
190	9.46	94.41	46.86	21.80	91.36	47.96	53.53	39.10	50.18	204.94	3.36	380.72	50.33	0.86	273.14
191	0.72	25.67	30.85	74.25	119.03	27.36	9.06	24.10	100.12	394.14	24.44	352.48	81.50	1.57	168.38
192	2.85	62.47	21.92	7.80	134.64	150.46	13.82	18.75	22.70	76.86	2.37	252.61	48.50	0.37	243.43
193	6.18	53.51	12.46	7.20	81.46	143.95	22.25	20.65	124.37	272.36	1.84	233.36	50.00		158.03
194	1.09	32.63	60.37	17.80	387.74	26.81	18.70	43.80	133.94	263.68	1.91	410.35	73.33	0.95	277.75

195	1.45	26.87	6.75	4.10	94.46	5.29	8.13	1.20	22.75	58.49	1.71	166.24	15.00	0.21	469.49
199	2.99	79.18	25.85	7.80	44.03	15.27	44.65	28.00	101.10	88.02	2.71	305.25	49.50	0.55	21.94
202	1.10	42.53	17.07	7.90	51.87	36.09	8.80	46.80	33.08	39.01	1.75	190.94	97.00	0.57	118.12
205	2.71	34.49	17.65	5.00	34.60	49.66	2.19	29.60	30.71	38.18	3.33	215.06	27.00	0.17	81.17
209	3.19	110.88	46.47	23.60	140.43	150.21	22.12	71.90	52.76	256.75	2.26	392.72	111.00	0.58	52.92
210	3.30	65.94	14.02	15.20	81.86	84.96	6.10	79.05	28.66	260.36	2.19	267.09	64.50	0.23	84.09
212	9.33	73.07	16.31	2.01	37.95	139.63	20.83	19.55	1.88	94.05	5.88	237.04	31.50	0.24	218.93
213	0.48	64.98	3.61	5.17	57.13	108.76	6.24	20.60	68.64	52.98	0.59	174.75	22.00	0.19	80.47
214	0.86	22.93	19.88	6.07	70.96	129.64	3.08	41.20	28.15	78.18	1.14	166.46	42.00	0.05	60.90
218	7.54	67.04	51.76	17.75	107.97	192.98	2.71	3.54	29.34	92.78	3.02	322.39	84.33		18.65
221	0.63	24.24	5.75	6.30	65.57	157.40	4.75	9.50	52.78	36.26	1.35	166.89	45.33	0.19	108.02
222	1.22	37.81	19.07	1.60	63.43	163.58	5.42	8.10	15.40	94.96	1.71	163.89	62.33	0.07	172.54
223	4.02	125.53	61.10	11.72	90.22	216.03	18.43	62.20	17.87	279.42	2.96	403.40	62.67	0.81	61.62
224	9.60	155.10	8.58	11.31	72.25	154.56	11.90	16.80	93.15	90.13	1.65	236.11	65.50	2.5	216.76
225	6.75	84.45	74.01	11.32	118.62	107.74	4.63	11.10	56.23	288.31	1.22	513.97	97.67	2.22	79.02
226	1.22	37.93	25.34	2.86	61.83	110.56	12.25	59.20	12.06	177.76	2.89	233.18	100.00	0.13	446.57
227	2.03	57.03	30.33	2.80	120.05	204.99	13.89	0.40	72.26	148.25	12.52	274.84	40.00	0.03	184.14
229	5.86	113.73	31.75	4.20	133.89	296.11	23.26	43.40	24.68	292.06	1.86	323.02	99.33	1.08	76.84
230	1.70	39.57	17.50	6.10	65.23	107.94	7.39	36.75	8.20	132.76	2.09	213.55	42.67	0.48	25.37
231	1.66	37.25	19.63	7.75	92.07	114.21	1.14	66.35	56.16	55.12	15.74	336.36	71.67	0.48	31.90
232	0.17	38.00	6.67	5.39	55.64	81.12	4.58	82.40	129.57	17.48	0.24	235.19	122.00	0.42	73.94
234	0.09	13.64	5.33	1.20	50.96	108.70	5.57	30.20	33.29	52.20	0.30	218.04	86.50	1.41	101.47
235	1.82	31.34	56.42	5.00	88.81	223.55	3.43	69.00	7.05	165.36	5.26	318.85	181.00	0.32	225.97
236	6.78	134.24	63.01	7.90	106.36	311.42	45.10	82.00	58.73	459.14	1.80	496.68	76.33	0.65	333.47
237	6.10	94.90	27.05	3.77	154.1	257.76	1.55	27.40	97.85	168.27	8.84	404.47	84.33	0.54	314.82

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238	1.51	54.20	71.76	12.70	212.40	194.80	3.93	30.60	6.20	271.83	4.95	425.69	77.50	0.25	161.52
239	3.99	83.08	18.85	4.70	200.49	231.04	7.43	54.40	9.22	100.91	4.78	302.58	74.00	0.95	219.67
240	1.59	166.15	36.80	9.80	145.44	297.56	20.35	48.00	33.66	129.03	1.25	424.20	83.00	0.93	197.46
242	13.34	108.22	34.42	6.10	138.03	101.92	22.49	16.20	10.44	88.30	1.80	162.55	37.00	0.86	21.81
243	3.06	47.91	21.28	10.30	188.55	233.34	3.78	61.80	34.37	63.08	4.62	291.64	71.50		68.24
244	3.20	28.50	17.29	8.41	103.86	161.95	50.19	34.00	89.00	68.36	1.46	376.27	71.00	4.63	24.63
246	7.37	64.60	19.58	7.33	74.45	90.98	3.31	55.20	84.30	53.87	4.46	365.68	81.50	1.62	233.40
248	2.00	44.69	27.14	6.06	33.64	152.93	4.00	43.10	54.13	85.56	1.19	180.29	77.50	1.36	17.36
249	0.85	31.19	14.07	8.09	51.65	102.45	5.73	24.80	84.60	44.52	2.02	222.95	49.50	1.79	21.00
250	9.48	70.72	27.93	18.93	60.86	44.92	0.31	14.05	19.73	109.99	1.56	252.74	31.00	1.55	79.55
251	2.99	36.03	22.83	12.53	51.88	192.02	12.11	72.80	7.44	81.22	2.63	354.06	40.50	0.88	178.48
254	4.06	74.56	67.44	8.85	91.26	223.98	26.54	19.00	36.05	332.46	1.30	284.39	68.75	1.08	166.37
255	1.01	53.87	27.10	9.70	61.75	132.49	13.96	82.40	41.74	133.79	2.85	259.58	52.00	2.15	23.82
259	1.42	74.99	88.60	7.91	56.32	26.68	1.14	14.45	45.41	93.08	1.38	309.58	78.00	0.86	65.42
260	0.76	15.78	17.35	6.95	107.70	44.45	1.50	23.90	25.42	117.51	0.89	224.08	46.50	0.3	62.19
261	1.94	52.41	29.00	13.87	77.79	202.82	13.67	39.80	28.88	170.53	2.02	287.13	38.50	1.12	114.68
264	0.76	24.34	18.68	25.79	67.43	221.72	2.47	19.70	55.28	64.26	1.26	153.13	22.50	0.26	1.58
266	8.26	78.84	26.00	9.18	94.56	173.61	14.34	17.00	144.81	169.89	1.47	341.64	22.50	1.32	41.91
268	0.42	32.12	14.62	6.26	29.09	159.04	0.45	12.60	59.89	132.37	1.19	210.38	27.00	0.87	74.72
269	2.35	61.03	20.41	13.40	123.90	165.13	24.12	16.80	89.58	119.07	1.49	381.76	34.33	0.1	346.33
270	6.01	61.67	74.93	6.30	151.21	149.06	8.20	33.20	80.94	197.62	2.32	352.41	23.00	0.95	126.12
271	2.72	56.31	28.36	5.55	113.80	220.67	6.81	12.70	53.71	62.40	1.48	221.41	26.50	2.14	22.61

272	1.56	42.35	25.11	20.66	60.75	237.24	12.42	19.90	216.50	202.38	1.61	269.15	44.00	1.79	15.81
273	2.80	59.40	32.51	17.95	181.54	91.34	4.50	23.20	96.70	52.94	2.90	261.64	29.50	1.53	22.14
274	0.24	24.52	9.66	8.30	53.40	123.58	3.32	0.70	210.23	74.81	1.33	169.58	39.00	0.6	49.81
275	2.34	50.55	26.97	13.89	57.87	164.17	6.99	66.80	51.22	161.48	1.35	253.60	69.00	4.33	48.23
279	1.03	92.76	44.77	6.22	67.68	171.75	32.50	45.15	47.10	241.26	1.78	365.43	65.75	1.77	307.58
281	0.40	3.73	20.37	6.33	7.52	150.88	1.94	19.00	6.75	21.19	2.54	343.80	14.50	0.2	44.28
282	0.47	9.80	20.00	1.84	37.38	59.20	4.41	34.50	2.92	49.05	2.45	146.70	20.50	0.4	125.05
283	0.40	3.65	8.47	1.50	44.82	210.55	3.30	23.20	9.74	18.69	2.91	167.72	19.50	0.38	3.84
285	1.95	46.41	40.43	9.08	53.45	119.09	12.71	74.50	34.65	88.55	1.46	276.73	19.00	1.56	31.63
288	3.11	89.90	40.86	63.30	452.38	514.96	25.70	20.40	73.70	1425.86	2.75	670.88	46.00	2	737.33
293	3.53	57.50	35.42	45.93	232.75	774.53	6.52	52.80	72.20	760.60	2.83	521.90	71.40	1.26	124.50
295	0.43	12.31	32.18	23.25	93.39	560.10	7.43	120.20	44.75	44.30	1.15	289.06	43.33	0.46	9.88
296	0.35	8.97	12.01	3.17	9.18	231.43	5.30	27.93	8.26	18.57	2.92	169.34	20.00	0.58	64.72
297	4.66	33.85	5.56	4.47	103.33	414.61	5.89	22.60	70.05	59.90	1.32	309.21	30.00	0.19	14.63
298	3.79	23.09	42.95	6.90	31.10	365.27	13.86	31.70	49.02	116.28	42.01	341.50	46.50	0.26	35.58
299	7.05	70.01	20.42	12.70	43.15	82.80	33.92	26.50	99.64	123.30	2.50	440.76	62.00	0.84	28.47
300	5.43	32.53	42.84	6.70	111.74	75.92	34.21	48.60	57.90	63.70	1.47	328.23	32.50	0.32	44.43