

# **Drug Metabolizing Enzymes**

**Technical Resource Guide**

**SECOND EDITION**





<b>Contents</b> .....	<b>iii</b>
<b>Foreword to the Second Edition</b> .....	<b>v</b>
<b>Introduction</b> .....	<b>1</b>
<b>Drug Metabolizing Enzymes</b> .....	<b>3</b>
Oxidative Drug Metabolizing Enzymes .....	3
<i>Cytochrome P450</i> .....	3
<i>Flavin Monooxygenases (FMOs)</i> .....	4
Conjugative Drug Metabolizing Enzymes .....	5
<i>UDP glycosyltransferases (UGTs)</i> .....	5
<i>Glutathione transferases (GSTs)</i> .....	6
<i>Sulfotransferases (SULTs)</i> .....	6
<i>N-acetyl Transferases</i> .....	7
<b>Sources of Drug Metabolizing Enzymes</b> .....	<b>7</b>
Natural Drug Metabolizing Enzymes .....	7
<i>Human Liver Microsomes (HLMs)</i> .....	7
<i>Human Hepatocytes and Liver Slices</i> .....	8
<i>Recombinant Drug Metabolism Enzymes</i> .....	8
<i>Purified Recombinant DMEs</i> .....	9
<i>Overexpression of DMEs in Insect Cells</i> .....	11
Applications for Recombinant DMEs .....	11
<i>Isozyme Identification</i> .....	12
<i>Determination of Kinetic Parameters</i> .....	13
<i>Inhibitor Screening</i> .....	13
<i>Synthesis of Metabolites</i> .....	13
<i>High-Throughput Screening</i> .....	14
<b>Key Literature</b> .....	<b>17</b>
<b>Drug Metabolism Methods</b> .....	<b>19</b>
<b>Fluorescence-based High-throughput CYP450 Assays</b> .....	<b>20</b>
<b>Vivid® CYP450 Screening Kits</b> .....	<b>20</b>
Introduction .....	20
Kit Components .....	21
Storage and Stability .....	21
Assay Theory .....	22
Materials Required but not Supplied .....	23
Vivid® CYP450 High-throughput Screening Assay Protocol .....	23
<i>Basic Assay Protocol Outline</i> .....	24
<i>Reconstitution of the Fluorescent Standard and Vivid® CYP450 Substrate</i> .....	25
<i>Pre-mixing Reconstituted Vivid® CYP450 Substrate and NADP<sup>+</sup></i> .....	26
<i>Master Pre-Mix</i> .....	27
<i>Kinetic Mode HTS Assay Procedure (Recommended)</i> .....	28
<i>End-Point HTS Assay Procedure</i> .....	29
References .....	31
Appendix I. Preparing the Standard Curve .....	31
Appendix II. Suggested Protocol for the Analysis of Results .....	32
Appendix III. Commonly used CYP450 Inhibitors (Stop Reagent) .....	33
<b>Other Fluorescence-based Assays for P450s</b> .....	<b>34</b>
<b>HPLC-based CYP450 Catalytic Assays</b> .....	<b>37</b>
Outline of the Reaction .....	41
Standard Curve .....	41
Additional Notes .....	41

<b>CYP450 Assays for Additional Substrates</b> .....	<b>42</b>
CYP1A2 Ethoxyresorufin Deethylation Assay .....	42
Erythromycin N-demethylation Assay using CYP3A4 .....	43
<i>Reagents</i> .....	43
<i>Procedure</i> .....	43
<i>For Sample Assays Using RECO® System</i> .....	43
<i>Standard Curve</i> .....	43
<i>References</i> .....	43
<b>UDP-Glycosyltransferase Catalytic Assays</b> .....	<b>44</b>
Other Reagents Required .....	44
Experimental Design and Applications .....	45
Summary of the UGT Reaction Conditions .....	45
Assay Controls .....	45
Incubations .....	46
Glucuronide Detection .....	46
<i>TLC Analysis</i> .....	46
<i>Organic Extraction</i> .....	46
References .....	47
<b>Sulfotransferase Catalytic Assays</b> .....	<b>47</b>
Other Reagents Required .....	48
SULT Isozyme-specific Reagents .....	48
Experimental Design .....	48
<i>Single Reaction Protocol</i> .....	48
Assay Controls .....	49
SULT-specific Conditions for Probe Substrates and Additives .....	49
Calculation of SULT-Specific Activity .....	50
References .....	50
<b>Glutathione S-transferase Catalytic Assays</b> .....	<b>50</b>
Protocol Using Invitrogen's Recombinant GSTs .....	50
<i>Reagents Required</i> .....	50
<i>Experimental Design</i> .....	50
<i>Reference</i> .....	51
<b>Considerations for Kinetic and Inhibitor Studies with DMEs</b> .....	<b>51</b>
V <sub>max</sub> and K <sub>m</sub> Determination .....	51
References .....	55
Inhibition studies .....	56
<b>Inhibition of CYP450 Activity Using Antibodies</b> .....	<b>57</b>
Reagents .....	57
Procedure .....	57
References .....	57
<b>CYP450 Carbon Monoxide Binding Assay</b> .....	<b>58</b>
Reagents .....	58
Procedure .....	58
Notes .....	59
References .....	59
<b>Products</b> .....	<b>61</b>

# FOREWORD

## to the Second Edition

Since the First Edition of this Guide was printed in 1996, the use of recombinant enzymes for in vitro drug metabolism studies has evolved from something viewed with healthy skepticism to a widespread practice with significant impact on the selection of lead compounds. For much of this we have to thank those who pioneered the cloning and heterologous expression of DMEs. There are too many to list, but it is fair to say that the two developments that had the biggest impact on Invitrogen's CYP450 production methods were the cracking of the *E. coli* CYP450 expression impasse by Henry Barnes in Mike Waterman's group, and the successful co-expression of CYP450 and NADPH-reductase by Caroline Lee while at Glaxo-Wellcome. These two systems form the foundation for a large percentage of the basic and applied research on CYP450 biochemistry over the last several years.

The ability to probe an isolated CYP450 isozyme in well-defined in vitro reactions has led to the use of high-throughput approaches for defining the metabolic profile of chemical libraries, which has greatly improved the prospects of developing predictive models for drug metabolism. Improvements in fluorescent assay methods—pioneered by Danny Burke more than 25 years ago—are enabling screening of entire libraries for interaction with individual CYP450 isozymes. Because of these efforts, in silico screening will likely be the norm for pharmacokinetic profiling of drug libraries in the future. Nevertheless, as evidenced by recent reports of multiple substrate binding and substrate-dependent inhibition, which create additional challenges on the computational side, the ever-recalcitrant CYP450s are not going to make things easy.

Another big change since the First Edition is the dawning realization of the impact of conjugative enzymes on overall drug metabolism. Traditionally relegated to a backseat to the CYP450s, their role in primary metabolism and clinical outcome has been under-appreciated. However, the number of examples of first-pass metabolism catalyzed by conjugative enzymes is increasing. Concurrently, the tools for their in vitro analysis—recombinant expression methods and assay methods—are improving. These developments will lead to a focused pharma effort to better understand how the conjugative enzymes fit into the overall drug metabolism picture. Invitrogen is working hard to accelerate these efforts, just as we have with the CYP450s.

While the changes that will occur in this field between now and the Third Edition of the Guide are sure to be as exciting as they are currently unknown, we sincerely hope that you will find the Second Edition and Invitrogen's products and expertise useful in your research efforts today.



# Introduction

<b>Introduction</b> .....	<b>2</b>
<b>Drug Metabolizing Enzymes</b> .....	<b>3</b>
Oxidative Drug Metabolizing Enzymes .....	3
<i>Cytochrome P450</i> .....	3
<i>Flavin Monooxygenases (FMOs)</i> .....	4
Conjugative Drug Metabolizing Enzymes .....	5
<i>UDP glycosyltransferases (UGTs)</i> .....	5
<i>Glutathione transferases (GSTs)</i> .....	6
<i>Sulfotransferases (SULTs)</i> .....	6
<i>N-acetyl Transferases</i> .....	7
<b>Sources of Drug Metabolizing Enzymes</b> .....	<b>7</b>
Natural Drug Metabolizing Enzymes .....	7
<i>Human Liver Microsomes (HLMs)</i> .....	7
<i>Human Hepatocytes and Liver Slices</i> .....	8
<i>Recombinant Drug Metabolism Enzymes</i> .....	8
<i>Purified Recombinant DMEs</i> .....	9
<i>Overexpression of DMEs in Insect Cells</i> .....	11
Applications for Recombinant DMEs .....	11
<i>Isozyme Identification</i> .....	12
<i>Determination of Kinetic Parameters</i> .....	13
<i>Inhibitor Screening</i> .....	13
<i>Synthesis of Metabolites</i> .....	13
<i>High-Throughput Screening</i> .....	14
<b>Key Literature</b> .....	<b>17</b>

## Introduction

The “drug metabolizing enzymes” (DMEs) are a diverse group of proteins that are responsible for metabolizing a vast array of xenobiotic compounds including drugs, environmental pollutants, and endogenous compounds such as steroids and prostaglandins (1). From an enzymological point-of-view, they are most noted for their broad substrate specificity; some members of the cytochrome P450 (P450 or CYP450) and flavin monooxygenase (FMO) families are known to metabolize more than 50 structurally diverse compounds. Understanding the structure-activity relationships for the DMEs and their substrates is an important area of research that impacts on pharmacology, toxicology, and basic enzymology. The use of recombinant DMEs will play an increasingly important role in these efforts.

Conceptually, the drug metabolizing enzymes are divided into two groups. Oxidative drug metabolizing enzymes, which include CYP450s and FMOs, catalyze the introduction of an oxygen atom into substrate molecules, generally resulting in hydroxylation or demethylation. The conjugative enzyme families include the UDP-glycosyltransferases (UGTs), glutathione transferases (GSTs), sulfotransferases (SULTs), and N-acetyltransferases (NATs) (1, 2). The conjugative drug metabolizing enzymes catalyze the coupling of endogenous small molecules to xenobiotics that usually results in the formation of soluble compounds that are more readily excreted.

Invitrogen offers a broad line of drug metabolism products, which are listed in **Table 1** and in the Products Section at the end of this guide.

**Table 1 – Invitrogen’s drug metabolism products**

<b>Cytochrome P450</b>	<b>Isozymes Available</b>
Insect Cell BACULOSOMES® Reagents	1A2, 2B6, 2C9, 2C19, 2D6, 3A4, 3A5
<i>E. coli</i> -expressed Purified Enzymes	1A2, 2C9, 2C19, 2D6, 2E1, 3A4
RECO® System	1A2, 2C9, 2C19, 2D6, 2E1, 3A4
<b>CYP450 Accessory Proteins</b>	<b>Isozymes Available</b>
NADPH-P450 Reductase	Recombinant Human
Cytochrome <i>b<sub>5</sub></i>	Recombinant Human
<b>UDP-Glycosyltransferases</b>	<b>Isozymes Available</b>
Insect Cell BACULOSOMES® Reagents	1A1, 1A3, 1A6, 1A7, 1A10, 2B7
<b>Sulfotransferases</b>	<b>Isozymes Available</b>
Insect Cell Cytosolic extracts	1A1*2, 1A2*1, 1A3, 1E, 2A1
<b>Glutathione Transferases</b>	<b>Isozymes Available</b>
<i>E. coli</i> -expressed Purified Enzymes	A1-1, M1-1, P1-1
<b>Vivid® CYP450 Screening Kits</b>	<b>Isozymes Available</b>
Vivid® Red Screening Kit	2C9, 3A4
Vivid® Green Screening Kit	2C9, 3A4
Vivid® Blue Screening Kit	1A2, 2C9, 2C19, 3A4
Vivid® Cyan Screening Kit	2D6
<b>Antibodies</b>	<b>Isozymes Available</b>
P450 Antibodies, Monoclonal	1A1, 1A2, 2A6, 2B6, 2C8/9/18/19, 2D6, 2D6i, 2E1, 3A4/5
Sulfotransferase Antibodies, Polyclonal	1A3, 1E, 2A1

1. Guengerich, F.P. (1995) Cytochrome P450: Structure, Mechanism, and Biochemistry (Second Edition), Chapter 14, edited by Paul R. Ortiz de Montellano, Plenum Press, New York.

2. Parkinson, A. (1996) Toxicology: The Basic Science of Poisons (Fifth Edition), Chapter 6, edited by Curtis D. Klassen, McGraw-Hill.

## Drug Metabolizing Enzymes

### Oxidative Drug Metabolizing Enzymes

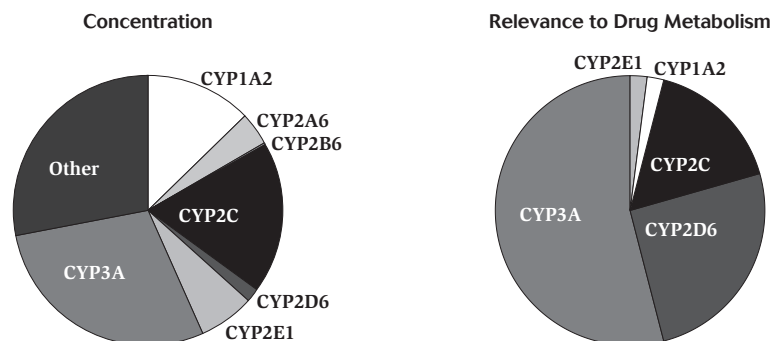
#### Cytochrome P450

CYP450 enzymes in the liver catalyze the initial step in the biotransformation of xenobiotic compounds, including most drugs. These enzymes are members of a large family of mixed-function oxidases that catalyze the introduction of an oxygen atom into substrate molecules, often resulting in hydroxylated or dealkylated metabolites.

More than fifty CYP450 isozymes are known to exist in humans (3) and they have been classified into 17 families and 39 subfamilies based on amino acid sequence similarities. Proteins from the same family are greater than 40% identical at the amino acid level, while those in the same subfamily are greater than 55% identical (3). In the standard nomenclature, the family is designated by a number followed by a letter designation for the subfamily, and a second number that identifies the individual member of that subfamily.

The bulk of drug metabolisms are carried out by a few members of the CYP1, 2, and 3 families and occurs primarily in the liver, which contains the highest concentration of CYP450 in the body. However, the importance of extrahepatic metabolism in tissues such as the intestine and lung is also recognized.

The xenobiotic metabolizing P450s are approximately 50 kDa proteins anchored in the endoplasmic reticulum (ER) by a single transmembrane helix in the N-terminus. Cell fractionation using differential centrifugation results in particulate preparations enriched in endoplasmic reticulum, commonly referred to as microsomes. Detailed examination of microsomal fractions from many different individuals has demonstrated significant variability in expression patterns of individual isozymes, however some generalizations are possible (1,4). On average, 70% of the P450s expressed in adult human liver consist of the following isozymes: 1A2, 2A6, 2B6, the 2C subfamily (2C8, 2C9, 2C18, and 2C19), 2D6, 2E1, and the 3A subfamily (3A4 and 3A5) (1, 4). **Figure 1** shows the concentrations of individual CYP450 enzymes in 60 samples of human liver microsomes and illustrates the percentage of drugs metabolized by the different CYP450 families. CYP3A4, 2D6, 1A2 and the 2C isozymes metabolize most of the drugs for which a specific isozyme has been identified (2). As **Figure 1** illustrates, the importance of a particular CYP450 isozyme in drug metabolism is not necessarily a function of its relative abundance in the liver. For example, CYP2D6 represents only about 2% of total hepatic CYP450, but metabolizes almost as many drugs as the most abundant CYP450, CYP3A4, which generally makes-up about 30% of the total CYP450 enzymes in human liver.



**Figure 1** — On the left, the spectrally determined concentration of individual P450 enzymes (from 60 samples of human liver microsomes) is displayed. These data are derived from Shimada *et al.*, (1994). Please note that CYP2C includes CYP2C8, 2C9, 2C18, 2C19 and allelic variants such as CYP2C10, while CYP3A includes CYP3A4, 3A5, 3A7 and allelic variants such as CYP3A3. On the right, the relevance of individual P450 enzymes to drug metabolism (measured by the percentage of drugs metabolized by a specific type of P450 relative to other P450 enzymes) is depicted.

1. Guengerich, F.P. (1995) Cytochrome P450: Structure, Mechanism, and Biochemistry (Second Edition), Chapter 14, edited by Paul R. Ortiz de Montellano, Plenum Press, New York.
2. Parkinson, A. (1996) Toxicology: The Basic Science of Poisons (Fifth Edition), Chapter 6, edited by Curtis D. Klassen, McGraw-Hill.
3. Nelson, D.R. (1999) *Arch. Biochem. Biophys.* **369**:1-10.
4. Shimada, T., *et al.* (1994) *J. Pharmacol. Exp. Ther.* **270**:414-23.

Oxidation of organic molecules by P450s is quite complex (5), but the overall reaction can be represented simply by **Equation 1**.



An electron from NADPH is transferred via the flavin domain of NADPH-P450 reductase to the heme domain of the CYP450 where the activation of molecular oxygen occurs. Substrates react with one of the oxygen atoms and the other is reduced to water.

In some cases, the second electron can come from NADPH via cytochrome *b*<sub>5</sub> reductase and cytochrome *b*<sub>5</sub>. During *in vitro* reconstitution experiments, cytochrome *b*<sub>5</sub> can stimulate metabolism of various substrates by some CYP450 isozymes, notably 3A4, 2E1, and 2C9. However, the mechanism of this stimulation is not clearly understood. Apo-cytochrome *b*<sub>5</sub> was shown to be as effective as the holoenzyme in stimulating reconstituted CYP3A4 reactions, so at least in this instance, it does not appear to be playing a direct role in electron transfer (6). The most widely held hypothesis is that cytochrome *b*<sub>5</sub> acts allosterically to enhance the interaction between CYP450 and NADPH-P450 reductase, or it improves substrate binding.

Isolating CYP450s can be difficult and tedious, hampering the use of reconstituted CYP450 activity from human tissue. Recently, methods for heterologous expression of recombinant CYP450s using *E. coli* and baculovirus (BaV) have greatly enhanced the utility of these proteins for *in vitro* metabolism studies.

In vitro manufactures, in several different formats, the following CYP450s: 1A2, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5.

#### Flavin Monooxygenases (FMOs)

Flavin monooxygenases, like the CYP450 enzymes, are associated with the endoplasmic reticulum and catalyze the oxidation of organic compounds using molecular oxygen and NADPH as the source of electrons for the reduction of one of the oxygen atoms (**Equation 1**). However, they are mechanistically distinct from the CYP450s in that they react with oxygen and NADPH in the absence of substrate to form a 4 $\alpha$ -hydroperoxy flavin enzyme intermediate. Thus, the FMOs exist in an activated form in the cell, and their interaction with a nucleophilic group such as an amine, thiol, or phosphate, is all that is required for completion of the catalytic cycle (7).

The capacity to remain stable while poised in an activated state is a possible explanation for the extremely broad substrate specificity of the FMO isozymes. It has been proposed that essentially all of the energy required for catalysis is captured in the oxygen-activated intermediate, and that alignment or distortion of the substrate molecules is not required (8). It follows that the active site of FMOs is much less sterically defined than for other enzymes. FMO3 is the most abundant form in human liver and is believed to be the dominant member of this enzyme family in terms of overall drug metabolism (7).

5. Ortiz de Montellano, P.R. (1995) *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Second Edition), Chapter 8, edited by Paul R. Ortiz de Montellano, Plenum Press, New York
6. Yamazaki, H., *et al.* (1996) *J. Biol. Chem.* **271**:27438-44.
7. Rettie, A.E. and Fisher, M.B. (1999) in *Handbook of Drug Metabolism*, pp131-147, edited by Thomas F. Woolf, Marcel Dekker, Inc, New York.
8. Ziegler, D.M. (1993) *Annu. Rev. Pharmacol. Toxicol.* **33**:179.

## Conjugative Drug Metabolizing Enzymes

### UDP glycosyltransferases (UGTs)

UDP glycosyltransferases catalyze the glucuronidation of xenobiotics at hydroxyl, carboxyl, amino, imino, and sulfhydryl groups using UDP-glucuronic acid as a donor molecule (**Equation 2**). In general, this generates products that are more hydrophilic and thus more readily excreted in bile or urine.



Although glucuronidation generally is classified as Phase II metabolism – the phase occurring after CYP450 dependent oxidative metabolism – many compounds do not require prior oxidation because they already possess functional groups that can be glucuronidated. Examples of first-pass metabolism catalyzed by UGTs include the UGT2B7-dependent glucuronidation of morphine (9) and the glucuronidation of 5-lipoxygenase inhibitors (anti-inflammatory) (10); in the latter case, glucuronidation was demonstrated to be the rate-limiting step for *in vivo* plasma clearance.

UGTs are 50-60 kDa integral membrane proteins with the major portion of the protein, including the catalytic domain, located in the lumen of the endoplasmic reticulum and a C-terminal anchoring region of 15-20 amino acids spanning the ER membrane (11). The aglycone-binding site is believed to be in the N-terminal portion the UGT polypeptide, which is the region of the protein that shows the greatest variability in sequence among UGT isozymes. The UDPGA binding domain is in the highly conserved C-terminal half of the protein. Although not a certainty, it has been hypothesized that association with lipid is required for UGT activity and may influence the access of aglycones to the active site.

Two UGT families—UGT1 and UGT2—have been identified in humans. Although members of these families are less than 50% identical in primary amino acid sequence, they exhibit significant overlap in substrate specificity (11). The members of the UGT1 family that are expressed in human liver, where the majority of xenobiotic metabolism takes place, includes UGT1A1, 1A3, 1A4, 1A6, and 1A9 (11). Although the UGT2 family has not been as extensively studied, it is known that UGT2B4, 2B7, 2B10, 2B11 and 2B15 are expressed in the liver (11). As is the case for other drug metabolizing enzymes such as CYP450s, inter-individual differences in UGT expression levels have been observed and linked to differences in drug responses (12).

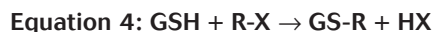
The human UGT1 family includes the major bilirubin metabolizing isoform (UGT1A1) and the isoform that preferentially conjugates planar phenols (UGT1A6). Isoforms in the UGT2 family metabolize a variety of endogenous steroid compounds, as well as xenobiotics. As with the CYP450s, classification of the UGTs based on substrate specificity is somewhat limited since there is a great deal of overlap in the biotransformation capacity for most of the human UGTs.

Invitrogen produces UGT1A1, 1A3, 1A6, 1A7, 1A10 and 2B7 in insect cell microsomes as BACULOSOMES® Reagents.

9. Coffman, B., *et al.* (1996) *Drug Metab. Dispos.* **25**:1-4.
10. Coffman, B., *et al.* (1997) *Drug Metab. Dispos.* **25**:1032-8.
11. Radominska-Pandya, A., *et al.* (1999) *Drug Metab. Rev.* **31**:817-99.
12. Weber, W. (1997) *Pharmacogenetics*, Oxford University Press, New York.

### Glutathione transferases (GSTs)

Glutathione transferases catalyze the formation of thioether conjugates between glutathione (GSH) and reactive xenobiotics by direct addition (**Equation 3**) or displacement of an electron-withdrawing group (**Equation 4**).



The major biological function of GSTs is believed to provide defense against electrophilic chemical species. The majority of GSTs are cytosolic homodimers composed of approximately 25 kDa subunits from one of four structural classes: Alpha ( $\alpha$ ), Mu ( $\mu$ ), Pi ( $\pi$ ), and Theta ( $\theta$ ). The  $\alpha$  isoform (GST A1-1) is restricted to a few tissues in mammals, including kidney, intestine, lung and liver. The  $\mu$  isoform (GST M1-1) is found in the liver, but relatively few other tissues. In contrast, the  $\pi$  isoform (GST P1-1) is widely distributed throughout the body, although it is notably absent in the liver. Additionally, GST P1-1 is abundant in most types of tumor cells.

Invitrogen manufactures purified GST A1-1, M1-1 and P1-1 expressed in *E. coli*.

### Sulfotransferases (SULTs)

Sulfotransferase enzymes catalyze the conjugation of sulfate groups onto a variety of xenobiotic and endogenous substrates that possess acceptor moieties such as hydroxyl and amine groups (**Equation 5**).



The cofactor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) is required for sulfonation by these enzymes. Although sulfonation generally causes molecules to lose their biological activity, several documented examples indicate that the addition of sulfate can lead to formation of highly reactive metabolic intermediates, such as minoxidil, and reactive electrophilic cations, such as sulfated N-hydroxy 2-acetylaminofluorene (13, 14). Several sulfotransferase enzymes with different biochemical properties have been characterized in animal and human tissue. Two general classes exist in tissue fractions: the cytosolic enzymes, which are considered important in drug metabolism; and the membrane bound enzymes, which are involved in the sulfonation of glycosaminoglycans and glycoproteins (15). The human cytosolic sulfotransferase isozymes function as homodimers of 32-35 kDa subunits. There are currently 10 known sulfotransferases in humans, five of which are known to be expressed in adult liver (SULT1A1, SULT1A2, SULT1A3, SULT1E and SULT2A1). It is expected that other new genes encoding sulfotransferases will be identified. The nomenclature of the different genes, their mRNA and protein products has recently been revised so that "SULT" is the accepted superfamily abbreviation (16). Allelic variants of sulfotransferase enzymes do exist and studying their frequency and functional role in drug disposition is a very active area of research.

Using insect cell cytosolic extracts, Invitrogen produces the following Sulfotransferases: 1A1\*2, 1A2\*1, 1A3, 1E and 2A1.

13. McCall, J., et al. (1983) *J. Med. Chem.* **26**:1791-3.

14. Miller, J.A. (1994) *Chem. Bio. Interact.* **92**:329-41.

15. Weinsilboum, R.M., et al. (1997) *FASEB J.* **11**:3-14.

16. Raftogianis, R.B., et al. (1997) *BBRC* **239**:298-304.

## N-acetyl Transferases

N-acetyltransferases (NATs) catalyze the biotransformation of aromatic amines or hydrazines to the respective amides and hydrazides (**Equation 6**) using acetyl coenzyme A as a donor. They also will catalyze the O-acetylation of N-hydroxyaromatic amines to acetoxy esters (**Equation 7**).



There are two known NAT isoforms in humans called NAT1 and NAT2; both are 33 kDa cytosolic proteins found in the liver. NAT1 is also expressed in many other tissues, whereas NAT2 is expressed only in the liver and gut. The two isoforms have different, but overlapping substrate specificities, with no single substrate appearing to be exclusively acetylated by one isoform or the other. Genetic polymorphisms for N-acetylation are well documented, and may play a role in the susceptibility of certain individuals to bladder and colon cancer, as the NATs are involved in both the activation and detoxification of heterocyclic aromatic amine carcinogens (12).

## Sources of Drug Metabolizing Enzymes

### Natural Drug Metabolizing Enzymes

#### Human Liver Microsomes (HLMs)

Microsomes are small vesicles isolated by centrifugation that were formed from the endoplasmic reticulum during cell disruption. Microsomal membrane preparations from human liver are the most extensively used source of enzymes for *in vitro* drug metabolism assays. They contain the major membrane-bound drug metabolizing enzymes present in the human liver, including CYP450s, FMOs and UGTs, and can be used to obtain a relatively broad metabolic profile for a test compound (17). Microsomes are also very simple to use for *in vitro* assays because they contain all of the protein and lipid components required for functional biotransformations. However, the presence of a mixture of DMEs and the wide variability in enzyme profiles between individual samples of liver microsomes makes them difficult to use for establishing the role of a specific enzyme in the metabolism of a compound. To overcome the problems associated with heterogeneity, elegant methods have been developed employing isoform-specific probe substrates and inhibitors in combination with isoform-specific antibodies (17). Using these tools, multiple liver samples (1-14) are exhaustively characterized with respect to the relative levels of the five or six most important CYP450 isoforms. Using a bank of “phenotyped” liver microsomes, correlations can be found between the rate of metabolism of a drug—or the inhibition of probe substrate metabolism—and the level of one or more specific CYP450 isoforms.

Phenotyped HLM banks are now routinely used to identify isozymes involved in the metabolism of new drugs. However, the use of correlative data from HLM banks still suffers from the uncertainty associated with any statistical analysis, the labor-intensive nature of the experimental design, and the complications in interpreting data when a compound is metabolized by more than one enzyme. In addition, the use of human liver tissue requires special safety precautions because they can be a source of blood-borne pathogens.

1. Guengerich, F.P. (1995) Cytochrome P450: Structure, Mechanism, and Biochemistry (Second Edition), Chapter 14, edited by Paul R. Ortiz de Montellano, Plenum Press, New York.
2. Parkinson, A. (1996) Toxicology: The Basic Science of Poisons (Fifth Edition), Chapter 6, edited by Curtis D. Klassen, McGraw-Hill.
3. Nelson, D.R. (1999) *Arch. Biochem. Biophys.* **369**:1-10.
4. Shimada, T., et al. (1994) *J. Pharmacol. Exp. Ther.* **270**:414-23.
5. Ortiz de Montellano, P.R. (1995) Cytochrome P450: Structure, Mechanism, and Biochemistry (Second Edition), Chapter 8, edited by Paul R. Ortiz de Montellano, Plenum Press, New York
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## Human Hepatocytes and Liver Slices

As with isolated human liver microsomes, human hepatocytes and liver slices are used to establish a complete metabolic profile of a compound (17). Neither can easily be used to identify the specific isoform(s) of the CYP450(s) responsible for the metabolism of a compound, and both must be prepared from fresh tissue. The main advantage of these systems is that they more closely mimic the *in vivo* enzyme environment than cell fractions or isolated enzymes. Recent improvements in the cryopreservation of human hepatocytes have greatly increased their utility for *in vitro* metabolism studies, especially for assessing enzyme induction. The long-term storage of liver slices is problematic; generally, they must be used within 5-7 days of their procurement (17).

## Recombinant Drug Metabolism Enzymes

In general, heterologous expression of soluble cytoplasmic enzymes is easier to achieve than for membrane bound proteins, and this holds true for the DMEs as well. The simplest expression system is *E. coli*, but when this is problematic, a variety of eukaryotic systems have been applied, including yeast, various mammalian cell lines, and baculovirus-infected insect cells. Obtaining high expression levels in *E. coli* and/or insect cells for the GSTs, SULTs and NATs (all soluble proteins) is relatively straightforward. However, the membrane bound CYP450s and UGTs present more of a challenge. As described in more detail in the following sections, bacterial expression of soluble, active CYP450s is possible when some N-terminal modifications are made; only the UGTs remain refractory to production in bacteria. Because of their predominant role in drug metabolism, significantly more effort has been directed toward CYP450 expression methods than the other DMEs. Some of the most successful approaches for heterologous CYP450 expression are summarized in **Table 2**. Yeast and various mammalian cells, most notably human lymphoblastoid cells were the first heterologous expression systems used as a practical source of recombinant CYP450s (18). However, for commercial production, these systems have largely been supplanted by *E. coli* and baculovirus-infected insect cells because of the high levels of expression possible (19).

**Table 2 – Sources of drug metabolizing enzymes for *in vitro* ADME studies**

Enzyme Source	Comments	Ref.
<b>CYP450s</b>		
Human Lymphoblastoid Cells	Low expression levels	18
Yeast ( <i>S. cerevisiae</i> )	CYP450 alone or CYP450/reductase	20
Baculovirus-infected Insect Cells	CYP450 alone or CYP450/reductase	21-23
<i>E. coli</i>	N-terminal modifications required; CYP450 or CYP450/reductase	24-32
<b>UGTs</b>		
COS cells	Low expression levels	33, 34
Human Embryonic Kidney (HK293) cells	Highest expression of active protein	35
Baculovirus-infected Insect Cells	Expression of active protein at a level less than 1mg/liter	34, 36, 37
<i>E. coli</i>	Expression of aglycone or UDPGA binding domains as fusion proteins	38, 39
<b>SULTs</b>		
Baculovirus-infected Insect Cells	Very high activity levels, purification unnecessary	37
<i>E. coli</i>	High level expression of soluble, active proteins	32, 40, 41
<b>NATs</b>		
<i>E. coli</i>	High level expression of soluble, active proteins	42
<b>GSTs</b>		
<i>E. coli</i>	High level expression of soluble active proteins	43

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## Purified Recombinant DMEs

From a manufacturing standpoint, *E. coli* provides the best expression system for producing enzymes for purification because of the high levels of enrichment possible. Invitrogen uses *E. coli* expression system to produce the key CYP450s and GSTs, as well as cytochrome *b<sub>5</sub>*, each in a highly purified form. Invitrogen is able to use baculovirus-infected insect cells to produce high levels of purified NADPH-P450 reductase. For the CYP450s, modifications to the N-terminal region along with extended induction times are critical for the expression of large amounts of properly folded holoprotein. The breakthrough in mammalian CYP450 expression in *E. coli* was made by Henry Barnes working on bovine CYP17A expression in Michael Waterman's laboratory (44). Changes in the first seven codons of the bovine CYP17A gene included silent mutations that increased AT richness (codons 4 and 5) and minimized potential mRNA secondary structure (codons 6 and 7). The use of GCT as the second codon is based on the finding that this codon is preferred at position 2 for optimal *lacZ* expression in *E. coli* (44, 45). Similar approaches have since been successfully applied to numerous mammalian CYP450s (24-28). In most cases (1A2, 3A4, 3A5, 2C10 and 2D6, but not 1A1), replacement of the N-terminus of a CYP450-encoding sequence with a modified CYP17A sequence has led to successful expression (24-26, 28). The N-terminal modifications used for each of the bacterially expressed CYP450s produced at Invitrogen are shown in **Table 3**. The pCWori<sup>+</sup> vector (44), with its tandem *tac* promoters and unusual spacing between the ribosome-binding site and initiation codon (3 bases rather than the usual 8-12), has been used for all of the high-level CYP450 expressions to date. However, it is not known what, if any, elements of this vector are critical. More recently, periplasmic targeting and the use of low levels of antibiotics to induce stress response have been used to further increase CYP450 expression levels in *E. coli* (46).

**Table 3 – N-terminal modifications made to human CYP450 cDNAs to enable expression of soluble holoprotein in *E. coli* for production of purified CYP450s at Invitrogen Corporation. The amino acids colored in **BOLD** in the native sequences have been deleted; the **BOLD** amino acids in the modified sequences represent the changes from the native sequences. Note that Invitrogen's 2D6 clone also has a C-terminal 6X His motif. Most of these modified cDNAs were licensed to Invitrogen Corporation by F. Peter Guengerich.**

CYP450 Isozyme	Native N-terminus	Modified N-terminus	Ref.
1A2	M <b>ALS</b> Q <b>S</b> V <b>P</b> F <b>S</b> A <b>T</b> E <b>L</b> L <b>L</b> A <b>S</b> A <b>I</b> F <b>C</b> L <b>V</b>	M <b>ALL</b> A <b>V</b> F <b>L</b> F <b>C</b> L <b>V</b>	28
2C9	M <b>D</b> S <b>L</b> V <b>V</b> L <b>V</b> L <b>C</b> L <b>S</b> C <b>L</b> L <b>L</b> L <b>S</b> L <b>W</b> R <b>Q</b> S <b>S</b>	M <b>A</b> R <b>Q</b> S <b>S</b>	47
2C19	M <b>D</b> P <b>F</b> V <b>V</b> L <b>V</b> L <b>C</b> L <b>S</b>	M <b>ALL</b> A <b>V</b> F <b>L</b> V <b>L</b> C <b>L</b>	48
2D6	M <b>G</b> L <b>E</b> A <b>L</b> V <b>P</b> L <b>A</b> V <b>V</b> A <b>I</b> F <b>L</b>	M <b>A</b> L <b>E</b> A <b>L</b> V <b>P</b> L <b>A</b> V <b>I</b> V <b>A</b> I <b>F</b> L	27
3A4	M <b>A</b> L <b>I</b> P <b>D</b> L <b>A</b> M <b>E</b> T <b>W</b> L <b>L</b> L <b>A</b> V <b>S</b> L <b>V</b> L <b>L</b> Y <b>L</b>	M <b>ALL</b> A <b>V</b> F <b>L</b> V <b>L</b> L <b>Y</b> L	24
2E1	M <b>S</b> A <b>L</b> G <b>V</b> T <b>V</b> A <b>L</b> L <b>V</b> W <b>A</b> F <b>L</b> L <b>L</b> V <b>S</b> M <b>W</b> R <b>Q</b> V	M <b>A</b> R <b>Q</b> V <b>H</b>	25

24. Gillam, E.M.J., et al. (1993) *Arch. Biochem. Biophys.* **305**:123-31.
25. Gillam, E.M.J., et al. (1994) *Arch. Biochem. Biophys.* **312**:59-66.
26. Gillam, E.M.J., et al. (1995) *Arch. Biochem. Biophys.* **317**:374-84.
27. Gillam, E.M.J., et al. (1995) *Arch. Biochem. Biophys.* **319**:540-50.
28. Sandhu, P., et al. (1994) *Arch. Biochem. Biophys.* **309**:168-77.
43. Kolm, R.H., et al. (1995) *Protein Expr. Purif.* **6**:265-71.18.
- Crespi, C.L., et al. (1991) *Chem. Res. Toxicol.* **4**:566-72.
44. Barnes, H.J., et al. (1991) *Proc. Natl. Acad. Sci. USA* **88**:5597-601.
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46. Pritchard, M.P., et al. (1997) *Arch. Biochem. Biophys.* **345**:342-54.
47. Sandhu, P., et al. (1993) *Arch. Biochem. Biophys.* **306**:443-50.
48. Richardson, T.H., et al. (1995) *Arch. Biochem. Biophys.* **323**:87-96.

DMEs purified from *E. coli* appear to exhibit substrate specificities and catalytic capacities similar to those determined for native enzymes. However, in the case of the CYP450s, some significant differences in  $K_m$  values have been observed with some substrates (37). The advantages of using purified, recombinant DMEs include the lack of interfering enzyme activities present in microsomes or other crude enzyme preparations and the flexibility to control and optimize reaction components, especially for multi-enzyme systems. It is well established that the relative amounts of CYP450, NADPH-P450 reductase, and cytochrome  $b_5$ , as well as the type and amounts of lipids present all have an effect on the biotransformation capacity of reconstituted CYP450 systems. The ability to vary these parameters allows optimization of the system for specific applications. To overcome some of the complications of reconstituting CYP450 systems, Invitrogen has developed a simplified *in vitro* assay system. The RECO® System incorporates all of the protein, lipid, and small molecule components required for *in vitro* reconstitution of oxidative biotransformations (49). As shown in **Table 4**, the RECO® System makes purified proteins a viable alternative to microsomal preparations for *in vitro* drug metabolism assays by eliminating cumbersome reconstitution methods.

Invitrogen manufactures the RECO® System for the following CYP450 isoforms: 1A2, 2C9, 2C19, 2D6, 2E1 and 3A4.

**Table 4 – Comparison of standard P450 reconstitution method with simplified RECO® assay method. The RECO® enzyme premix contains fully functional P450/NADPH-P450 Reductase/Cytochrome  $b_5$  biotransformation complexes in a lipid environment**

Traditional Method for P450 Reconstitution	Novel RECO® Assay Method	
1. Prepare mixture of lipids in organic solvent	<b>Reconstitution Steps Eliminated</b>	
↓		
2. Evaporate organic solvent from lipids		
↓		
3. Sonicate lipids to effect liposome formation		
↓		
4. Combine liposomes, P450 3A4, or Cytochrome $b_5$ , and detergent		
↓	1. Combine RECO® enzyme and buffer premixes, water, and substrate	
5. Preincubate to allow complex formation		↓
↓		2. Preincubate at 37°C
6. Add substrate and other buffer components		
↓		3. Add NADPH to start reaction
7. Preincubate at 37°C		
↓		
8. Add NADPH to start reactions		

37. Invitrogen Corporation, unpublished.

49. Shaw, P.M., et al. (1997) *Arch. Biochem. Biophys.* **348**:107-15.

### Overexpression of DMEs in Insect Cells

The ability to express foreign proteins in insect cells infected with baculovirus has made it possible to produce microsomes (BACULOSOMES® Reagents) containing complete, functional membrane-bound DME systems for CYP450s and UGTs. By using co-infection with multiple viruses and/or dual promoter viruses, co-expression of NADPH-P450 reductase (and in some cases, cytochrome *b<sub>5</sub>*) with various CYP450s has been achieved. BACULOSOMES® Reagents are as simple to use as human liver microsomes, yet allow investigators to perform *in vitro* assays with a single CYP450 or UGT isozyme. The level of CYP450 expression in BACULOSOMES® Reagents is often higher than in human liver microsomes, especially for the isozymes that are present in low abundance in human liver. The kinetic parameters ( $V_{max}$  and  $K_m$ ) appear to be similar to those determined in human liver microsomes. In general, however, the  $K_m$  values are slightly lower than human liver microsomes and the  $V_{max}$  values are greater. This may result from differences in the membrane environment or the ratio of NADPH-P450 reductase to CYP450, which is approximately 8:1 in BACULOSOMES® Reagents and approximately 1:10 in HLMs. The UGTs have not been as carefully characterized as CYP450s, but where data is available, the baculovirus-produced enzymes appear to exhibit similar properties to the enzymes in their native environment.

### Applications for Recombinant DMEs

**Table 5** lists some common uses of recombinant CYP450 isozymes for *in vitro* metabolism studies and the formats produced by Invitrogen Corporation that are most useful for these applications. Other DMEs can be used in similar ways. However, simple, rapid assay methods are not as well developed for the conjugative enzymes as they are for the CYP450s.

**Table 5 – Typical *in vitro* ADME applications and suggested recombinant CYP450 isozyme formats produced by Invitrogen**

Application	Recombinant CYP450 Format
Isozyme Identification	BACULOSOMES® Reagents, RECO® System
Kinetic Analysis	BACULOSOMES® Reagents, RECO® System
Spectral Studies	Purified (from <i>E. coli</i> )
Structure/Activity	RECO® System, Purified
Inhibitor Screening	BACULOSOMES® Reagents, RECO® System
Metabolite Production	Whole Cells – BaV or <i>E. coli</i>

Ideally, a drug development team would like to have a detailed picture of the pathway and kinetics of a compound's metabolism in humans, including possible side effects such as CYP450 induction/inhibition and the generation of toxic metabolites, before beginning clinical trials. Gathering as much of this data as possible usually involves a combination of increasingly targeted assay systems. Whole animals are often used for initial toxicological assessment and the outcome of these experiments can prevent a compound from entering the next phase even before any metabolism work is done. CYP450 induction is examined immunochemically using cultured hepatocytes, whole animals, and liver slices in combination with analytical methods to determine the overall metabolic profile. The identification of

the major metabolite(s) gives some indication of the classes of DMEs likely involved, especially if the initial metabolic transformation is oxidative (CYP450 or FMO) or conjugative (UGTs, SULTs, GSTs). A bank of characterized HLMs is generally used to identify the specific isozymes involved by correlating the metabolism rates with individual CYP450 levels.

Even with the application of increasingly sophisticated analytical methods, there are obvious difficulties in using whole animals, cells, or cell fractions to obtain information on the specific biochemical events that comprise a compound's metabolism. Advances in the molecular genetics and biochemistry of the DMEs, and the need for greater efficiency in the drug discovery process are driving the development of new *in vitro* methods based on isolated recombinant DME isozymes. These methods have been used for screening thousands of compounds, and are amenable to integration into the early phases of the drug discovery process. Some of the ways in which recombinant CYP450s can be used for *in vitro* metabolism studies and the rationale for these are described in the following sections. The same general approaches can be applied to other DMEs, but in most cases, the methods are not nearly as well developed for the conjugative enzymes as they are for the CYP450s.

### Isozyme Identification

Identification of the major enzyme(s) involved in your specific drug's metabolism is perhaps the most important component of early studies. Once this is known, kinetic studies are done to obtain  $K_m$  and  $V_{max}$  values. These parameters are used to estimate *in vivo* clearance rates, a key determinant of therapeutic efficacy. Knowledge of the metabolism rate by a specific CYP450 alerts the drug discovery team to potential pharmacogenetic problems or drug-drug interactions. Genetic differences in CYP450 levels are a major cause of individual variability in response to therapeutics. For example, 8% of the Caucasian population are "poor metabolizers" of 2D6 substrates and can experience serious side effects when administered normal doses of drugs that are metabolized primarily by this isozyme (1, 2). Furthermore, some drug-drug interactions can cause serious side effects or death. The identification of the enzyme primarily responsible for the metabolism of a drug aids in the design of effective clinical studies used for assessing possible drug interactions.

Invitrogen has a number of monoclonal antibodies raised against CYP450s that can be used to inhibit specific CYP450 activity in HLM or CYP450 mixtures and identify CYP450 isoforms involved in a specific metabolic activity. Antibodies are currently available for the following CYP450 isoforms: 1A1, 1A2, 2A6, 2B6, 2C8/9/18/19, 2D6, 2D6i, 2E1 and 3A4/5.

For isozyme identification, recombinant DMEs are used most often to confirm the correlative data obtained from HLMs. However, the availability of a "full panel" of recombinant enzymes covering the major human liver CYP450s allows a more direct approach (*i.e.*, assaying for metabolism of a test compound by incubation with the isolated isozymes). Either BACULOSOMES® Reagents or purified CYP450s in the RECO® System are well suited for isozyme identification in a high-throughput screening format. This can be done by following substrate consumption or product formation using the same analytical methods used for HLM-based assays with each isozyme or by testing for inhibition of probe substrate turnover by the test compound (*i.e.*, competition assays). "Inhibitors" identified in competition assays must be subsequently checked for metabolism to determine whether they are also substrates. A more detailed description of these approaches, including the use of fluorescent assay methods, is provided in the Section on **High-Throughput Screening**.

1. Guengerich, F.P. (1995) Cytochrome P450: Structure, Mechanism, and Biochemistry (Second Edition), Chapter 14, edited by Paul R. Ortiz de Montellano, Plenum Press, New York.
2. Parkinson, A. (1996) Toxicology: The Basic Science of Poisons (Fifth Edition), Chapter 6, edited by Curtis D. Klassen, McGraw-Hill. 17.  
Wrighton, S.A., *et al.* (1993) *Drug Metab. Rev.* 25:453-84.

### Determination of Kinetic Parameters

Undesirable pharmacokinetics is frequently a factor in the failure of compounds in preclinical studies. The goal of *in vitro* studies is to determine the kinetic parameters ( $K_m$  and  $V_{max}$ ) for a compound with the CYP450 isozyme of interest in order to obtain an estimate of the *in vivo* clearance rate. The problems with attempting to obtain accurate kinetic data from crude enzyme preparations such as microsomes are well documented. They include: metabolism of substrate by more than one isozyme, further modification of products (*i.e.*, conjugation), consumption of NADPH by contaminating redox enzymes, and binding of substrates or products to cell proteins or other macromolecules. From an enzymologist's point-of-view, the only way to obtain accurate kinetic data is with isolated enzyme systems. The recombinant CYP450 isozymes provide this capability. However, the possibility for differences in the binding or catalytic properties between CYP450 isozymes in their native environment and the recombinant enzymes (microsomes or purified) cannot be ignored. BACULOSOMES® Reagents are a good source of recombinant CYP450s for determining the kinetic parameters, as the kinetics observed with these preparations are very similar to those seen with HLMS for most of the compounds that have been tested. However, the ability to vary reaction components with the purified reconstituted CYP450 systems makes this format attractive as well.

### Inhibitor Screening

Inhibition of CYP450 isozymes has been shown to be the cause of some clinically relevant drug-drug interactions. The antifungal ketoconazole and macrolides (such as erythromycin) are competitive inhibitors of CYP3A4. The co-administration of these antibiotics with terfenadine, a CYP3A4 substrate, greatly decreases the terfenadine clearance rate, which can lead to lethal arrhythmias in some individuals. The use of recombinant CYP450 isozymes to screen for inhibitory compounds has already been incorporated into a high-throughput format by some of the major pharmaceutical companies. For instance, the co-expressed CYP3A4/Reductase system has been used to screen for CYP3A4 inhibitors using testosterone as a probe substrate coupled with robotically manipulated multi-column HPLC analysis (50). Additionally, the formation of metabolite-inhibitor complexes that have the potential to affect metabolism of other drugs can be detected spectrophotometrically using the purified enzymes from *E. coli* (51, 52). Fluorescence-based assays using either Vivid® fluorogenic substrates and BACULOSOMES® Reagents or the RECO® System are well-suited for this type of inhibitor screening, which is discussed in more detail in the section on **High-throughput Screening** and in the **Methods** Section.

### Synthesis of Metabolites

After identifying the CYP450 or other DME isoform(s) responsible for metabolism of a test compound, it is often desirable to produce large amounts of the metabolites for chemical evaluation, toxicity testing, and further metabolism studies. The use of recombinant CYP450 isozymes to catalyze stereo- and regio-specific reactions is increasingly being recognized as a valuable tool to aid the organic chemist during drug development (17, 53). Many of the reactions catalyzed by CYP450s involve the addition of an oxygen atom at a position far from any activating group, making traditional organic synthesis approaches untenable (53). In addition, the ability of CYP450s to catalyze stereo-specific additions can eliminate the need to separate racemic mixtures. Metabolite production is most efficient using cultures of *E. coli* or baculovirus-infected insect cells expressing the appropriate CYP450 isozyme and NADPH-P450 reductase. Invitrogen has performed numerous metabolite productions using the baculovirus approach with yields in the hundreds of milligrams.

17. Wrighton, S.A., *et al.* (1993) *Drug Metab. Rev.* **25**:453-84.

50. Tweedie, D. (1996) Proceedings of the Seventh North American ISSX Meeting.

51. Shet, M.S., *et al.* (1993) *Proc. Natl. Acad. Sci. USA* **90**:11748-52.

52. Peck, C.C., *et al.* (1993) *J. Amer. Med. Assoc.* **269**:1550-2.

53. Coon, M.J., *et al.* (1992) *FASEB J.* **6**: 669-73.

### High-Throughput Screening

**CYP450s**—A large number of pharmacologically active compounds synthesized in the discovery phase of pharmaceutical R&D are rejected because they interact with the metabolism of existing therapeutic drugs or because they have poor bioavailability caused by rapid metabolism. In many cases, this is because the compounds are either substrates or inhibitors of one or more cytochrome CYP450 isozymes. CYP450s and other DMEs are generally assayed by isolation and quantification of the metabolites produced from the parent compound. In most cases, this involves chromatographic techniques (usually HPLC) and in some cases phase separations. There are two major drawbacks to these assay methods. First, the need to isolate the reaction products makes the methods too cumbersome and time consuming for use in any type of high-volume assay format and precludes the collection of continuous kinetic data. Second, measurement of metabolites requires use of different assay methods for every substrate, raising an obvious technical barrier to screening diverse compounds for metabolism.

A “universal” assay method would be ideal in that it would allow direct quantification of metabolism rates for any substrate, allowing the determination of the key pharmacokinetic parameter ( $V_{max}/K_m$ ) for diverse compounds in a high-throughput format. The most obvious approach for achieving this is to monitor NADPH consumption, which theoretically should be stoichiometric with substrate turnover. However, this has not proven practical because the coupling between NADPH consumption and substrate turnover is variable depending on the substrate and is frequently as low as 20-30%. Measurement of oxygen consumption suffers from the same drawback; a significant percentage of the total oxygen consumed is diverted into reactive oxygen intermediates rather than metabolite and water.

For these reasons, the main approach that has been used for screening is competitive inhibition assays, in which inhibition of probe substrate turnover by the test compound is used to identify potential substrates and inhibitors. The hits from these competitive inhibition screens must be further evaluated to determine whether they are inhibitors or substrates for the indicated isozyme. A number of approaches have been developed for high-throughput screening of CYP450 inhibition. These techniques include rapid phase separation methods for isolating radiolabeled CYP450 2D6 metabolites (54), development of robotically controlled, multi-column HPLC separation systems to assay testosterone metabolism by CYP450 3A4 (50), the use of sensitive colorimetric reagents for quantitation of formaldehyde formation during CYP450-dependent demethylation reactions (55), and rapid LC/MS approaches for metabolite analysis. However, all of these approaches include relatively cumbersome post-reaction separation steps that limit their usefulness for a high-throughput screening format. The approach that shows the most promise for high-throughput inhibitor screens is the use of fluorescence assays, which can be done in a homogenous format (*i.e.*, they require no post-reaction separation steps).

Historically, fluorogenic CYP450 substrates have had either poor kinetics or the enzymatic products did not have the optical properties necessary to make large-scale screening affordable. If fluorogenic substrates were developed that could be metabolized efficiently by human CYP450 enzymes to generate highly fluorescent products, there would be several immediate benefits to drug screening outside of the obvious improvements in rapidly screening large numbers of compounds. Additional benefits would include the early detection of compounds with potential metabolic liabilities, the analysis of the structure/activity relationship(s) during compound-CYP450 interactions and rational design guidance for medicinal chemists during lead optimization.

50. Tweedie, D. (1996) Proceedings of the Seventh North American ISSX Meeting.

54. Rodrigues, A.D., *et al.* (1994) *Anal. Biochem.* **219**:309-20.

55. Queensberry, M.S. and Lee, Y.C. (1996) *Anal. Biochem.* **234**:50-5.

To this end, fluorescent substrates and assays have been developed. Aurora Biosciences' Vivid® Substrates, described in detail in the **Methods** Section, for all of the key human CYP450s, have been adapted to a multi-well format, thus providing the capability to screen large numbers of diverse chemicals for interaction with isolated recombinant CYP450 isozymes (56). The Vivid® CYP450 Fluorogenic Probe Substrates are now supplied in kits with Invitrogen's highly active and specific BACULOSOMES® CYP450 Reagents. Together, the Vivid® Fluorogenic Substrates and BACULOSOMES® Reagents, provide an ideal, non-radioactive method for studying of cytochrome P450 isoenzyme-drug interactions.

Vivid® Fluorogenic Substrates offer significant benefits over conventional probes, including a high turnover rate and superior fluorescent properties. They are metabolized to highly fluorescent products with superior extinction coefficients and superior aqueous fluorescence quantum yields. These probes excite in the visible light spectrum, which minimizes interference from test compounds and NADPH, as well as providing improved aqueous solubilities.

The CYP450-dependent metabolism of certain alkoxyresorufins and coumarin derivatives to products with different fluorescence spectra has been known for many years (57, 58). The most commonly used reaction was the dealkylation of ethoxy- and methoxy-resorufins by CYP1A isoforms. Fluorescent probes for all of the key human hepatic CYP450s have been identified and fluorescent inhibitor screens are increasingly becoming accepted as a valid approach for screening compound libraries. There is substantial overlap in the specificity of the various CYP450 isozymes for most of the fluorescent assay probes that have been developed; some useful combinations are described in the **Methods** section. A caveat of this approach is that it is becoming clear that the ability to detect competitive inhibition of a given CYP450 is dependent upon the probe substrate used. This is probably because the CYP450 binding pocket can accommodate more than one compound simultaneously. Thus, it may be necessary to run inhibitor screens for each CYP450 isozyme with multiple fluorescent probes that adequately cover the chemical space of potential inhibitors for the key hepatic CYP450 isozymes in order to avoid missing some interactions.

**UGTs**—There has not been nearly as much focus on development of high-throughput screening methods for the conjugative DMEs, although in most cases the technical challenges are similar to those faced with the CYP450s. In the case of UGTs, a coupled assay that quantifies UDP formation by enzymatic coupling to NADPH formation was demonstrated many years ago. However, the low wavelength used for NADPH detection would result in an unacceptable amount of interference from test compounds (59). A “universal” HPLC method for separation of radiolabeled glucuronides from [<sup>14</sup>C]-UDPGA was subsequently developed. While a substantial improvement over TLC-based separation methods, it still does not provide a truly high-throughput approach (60). Recently Invitrogen demonstrated the feasibility of a high-throughput approach for screening UGT substrates that does not require the use of radioactivity or the separation of products from reactants (61). The assay principle is based on the ability of glucuronide produced in UGT reactions to competitively inhibit a fluorescence based β-glucuronidase reporter reaction, as outlined in **Figure 2**. Because of the differences in the inhibition pattern observed for different glucuronides (data not shown), this approach is not quantitative, however it does serve as a rapid, qualitative screening method. Multiple techniques for performing fluorescence-based inhibition assays are described in the **Methods** Section.

56. Crespi, C.L., et al. (1997) *Anal. Biochem.* **248**:188-90.

57. Burke, M.D., et al. (1985) *Biochemical Pharmacol.* **34**:3337-45.

58. Buters, J.T.M., et al. (1993) *Biochemical Pharmacol.* **46**:1577-84.

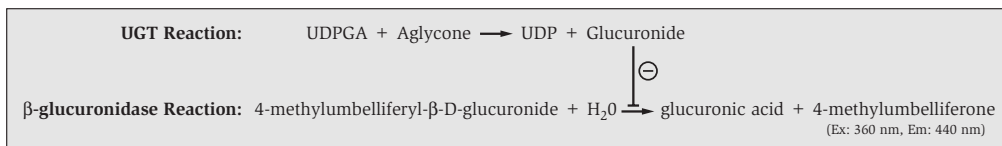
59. Mulder, G. (1975) *Biochem. J.* **151**:131-40.

60. Ethell, B. (1998) *Anal. Biochem.* **255**:142-7.

61. Trubetskoy, O.V. and Shaw, P.M. (1999) *Drug Metab. Dispos.* **27**:555-7.

**Other Conjugative DMEs**—GSTs can be screened for inhibitors using the colorimetric assay probe chlorodinitrobenzene, which serves as an acceptor for all of the known human isoforms. There are no high-throughput approaches available for NATs. However, a new, rapid, non-radioactive method for measuring sulfotransferase activity in 96-well plates has recently been described (62).

**Figure 2** — A schematic diagram of the detection of glucuronidation by coupling its formation to the inhibition of a fluorescent  $\beta$ -glucuronidase reporter reaction.



62. Frame L.T., et al. (2000) *Drug Metab. Dispos.* 28:1063-8.

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## Key Literature

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**Cytochrome P450: Structure, Mechanism and Biochemistry 1995 (Second Edition)** edited by Paul R. Ortiz de Montellano, Plenum Press, New York This is the definitive tome on cytochrome P450 structure, function and enzymology. Most of the information in the 1995 edition, especially enzymology, is still relevant despite the rapid progress in P450 structure over the over the past few years.

**Biotransformation of Xenobiotics: Chapter 6 from Casarett and Doull's Toxicology: The Basic Science of Poisons (Fifth Edition),** by Andrew Parkinson (1996), edited by Curtis D. Klassen, McGraw-Hill Dr. Parkinson has put together an extremely useful overview of the chemistry of xenobiotic metabolism in this Chapter. He systematically classifies and describes the chemistry and enzymology of each of the various types of oxidative and conjugative reactions involved in xenobiotic metabolism. He also supplies numerous examples to clarify his descriptions, including multi-step, branched pathways to illustrate the combinatorial nature of xenobiotic metabolism.

**Handbook of Drug Metabolism (1999)** edited by Thomas F. Woolf, Marcel Dekker, Inc, New York This book brings together several of the disparate areas relevant to drug metabolism and presents them from the practical perspective of people actively working in the field. It covers the fundamentals, from hepatic structure and function and pharmacokinetics, all the way through some of the *in vitro* methods used to assess metabolism during drug discovery.

**Pharmacogenetics (1997)** Wendell Weber, Oxford University Press, New York After a good overview of the basic concepts of pharmacogenetics, including an historical perspective, there are 200 pages of specific examples of genetically-linked variability to drug responses. For each of these, there is a brief explanation of the genetics and biochemistry behind the variable response.

**Cytochrome P450 Protocols (1998)** Edited by Ian R. Phillips and Elizabeth A. Shepard, Humana Press, Totowa NJ This book contains a wide variety of CYP450 methods, including purification of CYP450s from natural sources and hepatocytes and methods for analyzing of CYP450 gene expression and the detection of polymorphisms. The information is presented with minimal discussion, but in most cases, it includes all of the information actually required to carry out the technique, as well as cautionary notes on what can go wrong.

**Structural and functional studies of UDP-glucuronosyltransferases (1999)** Radomska-Pandya, A., Czernik, P.J., Little, J.M., Battaglia, E., Mackenzie, P.I., *Drug Metab. Rev.* 31:817-899 This article is a good source of information on the UGTs.

**P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature (1996)** Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., Nebert, D.W. *Pharmacogenetics.* 6:1-42. This reference contains the standard accepted nomenclature and phylogeny for CYP450s. The most recent additions and changes can be found on the CYP450 homepage, which is described below.

**The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence (1997)** Mackenzie, P.I., Owens, I.S., Burchell, B., Bock, K.W., Bairoch, A., Bélanger, A., Fournel-Gigleux, S., Green, M., Hum, D.W., Iyanagi, T., Lancet, D., Louisot, P., Magdalou, J., Chowdhury, J.R., Ritter, J.K., Schachter, H., Tephly, T.R., Tipton, K.F., Nebert, D.W. *Pharmacogenetics*. 7:255-69. The standard accepted nomenclature and phylogeny for the UGTs is described here.

**The P450 homepage:** <http://drnelson.utmem.edu/CytochromeP450.html> This page contains the most extensive analysis of CYP450 genetics and phylogeny available, including sequence databases, alignments, and phylogenetic trees. It is very carefully maintained and frequently updated to include newly discovered CYP450 genes from many different species, while removing those entries found to be artifacts. The P450 Homepage also contains links to other CYP450 sites and meetings.

**The Cytochrome P450 Drug Interaction Table:** <http://www.dml.georgetown.edu/depts/pharmacology/davetab.html> This reference contains entries for many of the known CYP450 substrate inhibitors and inducers, with many of the entries linked to the primary literature.

**Methods Enzymology, Volume 272 (1996) Cytochrome P450** This contains many useful protocols for CYP450 biochemistry, immunology and molecular biology.