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# Cytochrome P450 Protocols

## SECOND EDITION

*Edited by*

Ian R. Phillips
Elizabeth A. Shephard

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# Cytochrome P450 Protocols

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METHODS IN MOLECULAR BIOLOGY™

# Cytochrome P450 Protocols

*Second Edition*

Edited by

**Ian R. Phillips**

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# Preface

Cytochromes P450 (P450) comprise a large superfamily of proteins that are of central importance in the detoxification or activation of a tremendous number of foreign hydrophobic compounds, including many therapeutic drugs, chemical carcinogens, and environmental pollutants. Many of these enzymes are induced by the compounds they metabolize. In addition, genetic polymorphisms of P450 genes can lead to adverse drug reactions. Consequently, P450s are one of the most extensively studied groups of proteins, being investigated by researchers in fields as diverse as toxicology, pharmacology, genetics, environmental biology, biochemistry, and molecular biology. The wide range of techniques that have been applied to the P450s reflects the diverse backgrounds of the many researchers active in this field.

The second edition of *Cytochrome P450 Protocols* contains a collection of key “core” techniques for the investigation of P450s. Although the emphasis is on P450s of mammalian origin, many of the methods described are suitable for the investigation of P450s from any source. Also included in this edition are chapters on the flavin-containing monooxygenases (FMOs), another family of proteins that are important in the metabolism of xenobiotics, and that share several substrates in common with the cytochromes P450.

Each chapter is written by researchers who have been involved in the development and application of the particular technique to P450s or FMOs. Protocols are presented in a step-by-step manner, with extensive cross-references to notes that highlight critical steps, potential problems, and alternative methods. We hope that this format will enable researchers who have no previous knowledge of the technique to understand the basis of the method and to perform it successfully.

*Cytochrome P450 Protocols* begins with a chapter on P450 nomenclature and classification, which will serve both as an introduction to those new to the field and as a guide for more experienced workers wishing to name their pet P450. Although not formally divided into sections, the remaining chapters are grouped according to topic. These include methods for spectral analysis and purification of P450s; enzymatic assays of P450s and FMOs; expression of P450s and FMOs in heterologous systems; production and use of anti-peptide antibodies; transfection of hepatocytes for gene regulation studies; P450 reporter gene assays; *in situ* hybridization; analysis of genetic polymorphisms;

and P450 allele nomenclature, including a description of the P450 allele website. Because of the increasing importance of in vitro systems for pharmacotoxicology research, we have included several chapters on the preparation and culture of rodent and human hepatocytes and the production of bone marrow stem cells. The final chapters describe more specialized techniques for the generation of mice with targeted gene disruptions.

We are extremely grateful to all the authors who contributed so generously to this volume and to the *Methods in Molecular Biology* series editor, John Walker, for his guidance and forbearance.

***Ian Phillips***
***Elizabeth Shephard***

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# 1

## Cytochrome P450 Nomenclature, 2004

**David R. Nelson**

**Summary**

Aspects of cytochrome P450 (CYP) nomenclature are addressed. The rules for naming a P450 are outlined, though individuals should not name their own genes. The nomenclature is presented as a unifying principle to enhance communication across disciplines. Because of the historical nature of gene sequencing, sometimes names have to be changed, but this is kept to a bare minimum to avoid confusion in the literature. CYP names have now reached four digits owing to proliferation of CYP families in the fungi and lower eukaryotes. For example, *CYP5034A1* is from *Ustilago maydis*. P450 sequence motifs are described that are useful in making global alignments. CYP clans are defined as clusters of CYP families. The clan names are useful in describing higher-order evolution of the gene superfamily. The nomenclature of orthologs and pseudogenes is also discussed.

**Key Words:** Cytochrome P450; CYP; P450 clans; nomenclature; motifs.

### 1. Introduction

#### *1.1. Moving From Hundreds to Thousands of Sequences*

The previous publication of this chapter recognized 753 named P450 sequences in mid-1998. In September 2004, the Cytochrome P450 (CYP) count was 3811 and rapidly moving to 4000. Eukaryotic genomes are being sequenced in months, not years, and annotation has become the rate-limiting step. The nomenclature system for cytochrome P450, first devised in 1987, has become strained, but it is not broken ***(1)***. This system relies on evolutionary relationships as depicted in phylogenetic trees. There is an arbitrary 40% amino acid sequence identity rule for membership in a family and a 55% rule for membership in a subfamily. The actual decision to include a sequence in an existing group largely depends on how it clusters on a tree and not so much on the absolute percentage of identity, which is more or less a rule of thumb. Owing to the great diversity of P450s in insects, fungi, and bacteria, there is a need for additional layers of nomenclature, above the family/subfamily level. This is similar to the multiple levels of the Linnean classification scheme for species. The concept of clans has

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been introduced *(2,3)* as a level above family rank. It is possible that subclans or superclans may be needed, but the exact details of this are only now being discussed by those concerned with P450 nomenclature. The general Web repository for P450 nomenclature and sequence data is http://drnelson.utmem.edu/cytochromeP450.html.

### 1.2. Nomenclature Is Philosophy

The average person who needs to use a gene name does not consider how the name was arrived at, or what its implications are. Under the 40% rule, a new sequence submitted for naming by an individual may be 38% identical to an existing family such as CYP1, with three subfamilies. On a tree, this family may be well separated from its neighbors, so the new sequence seems to belong in the CYP1 family. In this case, it would become *CYP1D1*. However, someone under pressure for grant funding and tenure decisions, would strongly like to see the new sequence in a new family. A decision must be made based on the best interests of the nomenclature; this may disappoint an individual.

### 1.3. Nomenclature Is a Unifying Principal

Biochemists, geneticists, molecular biologists, and others who discover genes, often name those genes in an appropriate manner based on how they work or to what pathway they belong. Often, these are very useful names and well understood by researchers in these fields. Frequently, the gene has been named for a mutant phenotype before the gene sequence was discovered. *ERG5* and *ERG11* are two P450 genes in the ergosterol pathway of fungi, but they are also *CYP61A1* and *CYP51F1*. The Halloween genes disembodied (*dib*), phantom (*phm*), shade (*shd*), and shadow (*sad*) are required for ecdysone synthesis and its further metabolism in arthropods. They are embryonic lethal mutations in *Drosophila*. These genes are also Cyp302a1, Cyp306a1, Cyp314a1, and *Cyp315a1*, respectively *(4–6)*. Spook (*spo*) is a fifth Halloween P450 gene, but the sequence has not been revealed yet *(7)*. The two nomenclatures exist side by side and will be used by different groups for different audiences. There is nothing wrong with this. The Cyp names identify these genes as P450 genes. The numbers in the 300 range identify them as animal P450s. Furthermore, *Cyp302a1*, *Cyp314a1*, and *Cyp315a1* are in the mitochondrial clan, whereas *Cyp306a1* is in the CYP2 clan (*see* **Subheading 1.4.**). This information follows from the P450 nomenclature and shows relationships to other P450 genes in these clans. The CYP nomenclature should be cross-referenced when the phenotype-specific names are used.

### 1.4. Nomenclature May Change

Because names are assigned in historical order, often without the benefit of knowing whole genome P450 collections and/or related genes from other species, some names will need to be revised. When 455 P450s were named from the rice genome, 3 P450s from *Arabidopsis* were found to cluster in new locations on the phylogenetic tree *(8)*. *CYP709A1* and *CYP709A2* became *CYP735A1* and *CYP735A2*, respectively, because they clearly separated from the other CYP709 sequences. *CYP721B1* became *CYP734A1* for similar reasons. From the 272 *Arabidopsis* genes and pseudogenes to the total collection now of more than 1100 plant P450s, only three names were changed

in *Arabidopsis*. In the future, name changes in existing plant sequences should be even more rare or nonexistent, because the nomenclature stabilizes as the sequence space or diversity is more completely known.

### *1.5. Nomenclature Growing Pains*

The 3811 P450s named to date have exceeded the capacity of the original nomenclature system that was based on fewer than 100 P450 families in eukaryotes and an open-ended number in bacteria. The first 100 families were divided among animals (CYP1–49), lower eukaryotes (CYP51–69), and plants (CYP71–99). Bacteria began with CYP101 and could go upward from there. Plants were the first to break the system. The solution was to give three-digit CYP names, allowing for about 1000 new CYP families. The numbering scheme is similar to that of the first 100, except bacteria already were in the 100 and higher range. The four divisions were assigned as follows: bacteria, CYP101–299; animals, CYP301–499; lower eukaryotes, CYP501–699; and plants, CYP701–999. Bacteria, animals, and plants still fit this three-digit system, but lower eukaryotes have already broken the CYP699 limit (*CYP699A1* is from *Agaricus bisporus*, a mushroom). A four-digit system has been set up with similar ranges: bacteria, CYP1001–2999; animals, CYP3001–4999; lower eukaryotes, CYP5001–6999; and plants, CYP7001–9999. This historical naming system may induce some CYP name prejudice, with the one- and two- digit names occupying a privileged place in the eyes of some. *CYP1A1* from vertebrates may be accorded higher status than *CYP5034A1* from *Ustilago maydis*. This is not the intent of the nomenclature, but it may reflect where the early experimental efforts on P450 first uncovered these genes. For instance, there are no vertebrate P450s above *CYP51*.

## 2. Naming a New P450 Gene

New P450 genes should be submitted to the Committee on Standardized Cytochrome P450 Nomenclature for naming. That committee consists of David Nelson and any other expert needed in case of a tricky nomenclature problem. Usually, that means David Nelson will name the gene, and the sequence is kept confidential. Names should not be assigned by an author without help from this central committee in order to avoid inaccurate or duplicate names. One author named a rabbit gene *CYP12* because it hydroxylated its sterol substrate on the 12 position ***(9)***. However, CYP12 is a family of insect P450s found in mitochondria, so this gene was renamed *CYP8B1*. Because many confidential sequences are named, the only way to be sure one's sequence has not already been assigned a name is to consult the committee. In addition, as described in **Subheading 4.**, there are other considerations, such as orthology, that can affect a name. The 40% rule is a guideline that can be broken for good cause. It is very helpful to do BLAST searches and identify the best matches in the public database, but one should remember that there may be even better matches in the confidential set of P450s.

There is no limit to how many sequences one may submit for naming. The record as of this writing is all the P450s found in *Fusarium graminearum, Magnaporthe grisea*, *Aspergillus nidulans,* and *Neurospora crassa.* The *N. crassa* genes had already been

named, but the others included 341 P450 genes. It took more than a month to assign names to these genes.

The process that is used to name a gene is first BLAST searching known public P450s on the P450 BLAST server at http://132.192.64.52/p450.html. Thousands of P450s on this server have been assembled from genomic DNA or mRNA sequences, and sometimes overlapping expressed sequence tags have been used to make a complete gene. This set does not exist anywhere else in this useful form, because it is a curated nonredundant collection. The sequences are sorted by species or, in some cases, by larger taxonomic groups such as bacteria. Just a few searches here often can identify the best BLAST match in the named set of P450s and narrow the process down to a single family or subfamily. If the sequence does not match any known P450 at the 40% identity level or higher, then the sequence may be in a new family. This is often the case for fungal or bacterial sequences. It is never the case for vertebrate sequences, because no new families of P450s have been discovered in vertebrates for at least 5 yr.

If the sequence is in a new family, more extensive searches need to be done in Genbank for related sequences. It may be necessary to name a whole new family of sequences, rather than just the one submitted. Sometimes, a sequence that is from an animal or a plant may not match any families in those groups. It is a good practice to search fungal and bacterial sequences to detect a potential contamination. I have had to notify researchers conducting genome-sequencing projects of these problems.

Once a sequence is determined to belong to a family, the naming process can be completed quickly if the sequence has 55% or greater identity to a known sequence. If it is less, then the subfamily must be determined. New subfamilies are continually being found. They can be hard to name in the large families of CYP4, CYP6, and CYP71. The CYP4 family has 43 subfamilies. The first 24 use single letters of the alphabet, as in CYP4A, CYP4B, and so on. Once CYP4Z was reached (*CYP4Z1* is a human sequence), double letters were used for subfamilies, CYP4AA, CYP4AB, up to *CYP4AX2*, a silkworm sequence. This process will continue using CYP4BA, CYP4BB, and so on. Note that the letters *O* and *I* are not used for subfamily names to avoid confusion with one and zero. Potentially $24^2$, or 576, subfamilies are possible with double letters.

To assign a new subfamily in a complex family such as CYP4, a tree must be made to see how the new branch clusters with all the other subfamilies. Usually this is done with a subset of sequences, but it may include up to 160 sequences for detailed results. Typical tree-drawing programs do not make readable trees if more than 160 sequences are included, but this is usually not required. For final assignment, it may be necessary to make both UPGMA and neighbor-joining trees to get a good idea where the new sequence belongs. Sometimes a judgment call is needed to decide whether a new sequence should be given a new subfamily name or be included in an existing subfamily. Additional information such as intron-exon locations can be quite helpful. Branches/sequences with low bootstrap support may shift from place to place in different trees, which can be problematic in naming these sequences.

### 3. Benchmarks for P450 Sequence Alignment

The construction of a tree is dependent on the sequence alignment. The program ClustalW is often used for this purpose. Alignment of P450 sequences that are in the same family is quite good by this automated method, but more distant sequences will pose problems for even the best algorithm. Alignments should be checked by eye to ensure that the benchmarks for P450 sequences line up properly. If they do not, they will need to be manually adjusted.

These P450 benchmarks or motifs are few in the N-terminal region, and more abundant in the C-terminal from the I-helix to the end of the P450 molecule. Many of these benchmark regions are specific for families or higher taxonomic groups, such as bacterial or mitochondrial P450s that tend to cluster together. When aligning difficult sequences with low similarity to other sequences, pay particular attention to these benchmark regions. Alignment algorithms cannot use this information, and they are often quite inaccurate when it comes to distantly related P450 sequences. This is understandable when a bacterial sequence may be only 8 to 9% identical to a mammalian sequence.

At the N-terminal of many plant and animal P450s is a proline-rich sequence. It is often PPGP, but it may be (hydrophobic; hdr)PGP. This sequence is usually followed four residues later by P(hdr)(hdr)G(polar)(hdr), as in PIIGNL. This region follows the N-terminal membrane-anchor sequence, which is usually not conserved and cannot be aligned with any certainty. About 20 amino acid residues later, there is a common sequence, KYG or RYG. This tripeptide is present in *CYP51*, lanosterol-14α-demethylase, which is one of the more ancient eukaryotic P450s involved in cholesterol biosynthesis. It is also present in *CYP7*, *CYP8*, and *CYP19* sequences, but not in *CYP4* or *CYP52* (fatty-acid and alkane hydroxylases) nor in bacterial-like sequences. The two basic P450 forms are called E-like and B-like P450s for eukaryote and bacterial-like, respectively. A tree computed from representatives of all known bacterial CYP families showed a clear break into E and B branches, with only two sequence families falling outside this pattern. These two were *CYP152* and *CYP198*. *CYP152A1* is a known peroxygenase that uses hydrogen peroxide instead of molecular oxygen in its reaction (***10***). *CYP152A2* is the only known P450 from an anaerobe, *Clostridium acetobutylicum* (GenPept AAK81262). This P450 in an anaerobe may serve a protective role against hydrogen peroxide. This major substrate difference may explain why *CYP152* falls outside the E and B clusters. The function of *CYP198* (GenPept AAM42184) is unknown.

The ancient origin of the KYG motif is further confirmed by its presence in *CYP110* from *Anabaena*, a cyanobacterium. In modified form (ELG) it is also seen in *CYP11B* and *CYP102* ($P450_{BM-3}$) from *Bacillus megaterium*, where it occurs just before the b 1-1 strand. Both *CYP110* and *CYP102* are E-like bacterial P450s, suggesting that the fundamental split between E- and B-like P450 structures occurred before eukaryotes developed. For comparison of the E-like P450 crystal structure of *CYP102*, and the B-like P450 crystal structures of *CYP101* ($P450_{cam}$) and *CYP108* ($P450_{terp}$), *see* **ref. *11***.

In bacterial B-like P450s, near amino acid 50 there is a conserved trp residue, often seen in the pattern WXXT(R,K), as in WIATK or WLVTR. This is the first region to look for near the N-terminus of a new bacterial sequence. About 100 amino acid residues from the N-terminus of bacterial sequences, there is another region, around the C-helix, that is critical when aligning bacterial sequences with eukaryotic sequences. The motif is DPPXHXXXR. This motif corresponds to a C-helix sequence, WXXXR, conserved in most eukaryotic P450s. The R is often followed by another basic group, as in WREQRR. Some lower eukaryotic P450s (CYP53, 57, and 58) have an H instead of the W seen in most eukaryotes.

After the C-helix, there is a long nonconserved stretch with few alignment cues. One worth mentioning is the (N,D,S)(V,I,T)(V,I) sequence around positions 175–180 in eukaryotic sequences. This E-helix region can be diagnostic for some families, as in the 3A subfamily, where the sequence reads GAYSMD(V,I)I, or the 4A subfamily SLMTLDT(I,V). After this region, the next well-conserved area is the I-helix. From here (near position 300) the P450s are much more strongly conserved and alignments are much easier. The I-helix has a characteristic sequence A(A,G)X(E,D)T, in which T is part of the molecular oxygen-binding site. This region is not conserved in enzymes that use peroxides as substrates, such as allene oxide synthase (CYP74), because they do not have to bind molecular oxygen. Consequently, the CYP74 family is one of the most distant branches on the P450 tree. This may not reflect its true evolutionary history, because one of the key regions used in computing trees is not conserved.

Exactly 16 amino acid residues from the T previously mentioned is a conserved P. This P is present even in many bacterial sequences, so it is a good marker for the junction between the I and J helices. The sequence conservation continues for another 15 residues in eukaryotes to a highly conserved G or P residue. At this point, there seems to be some variation in sequence length in the region between the J-helix and the K-helix. The K-helix is the best conserved feature in P450s, with its invariant EXXR charge pair. About 22 amino acid residues beyond the K-helix R, there is an (aromatic)X(I,V,L)P(K,A)G sequence that spans the connection between the β-2-2 strand and the β-1-3 strand (nomenclature of Peterson and Graham-Lorence; *see* **ref. *11***). The P(K,A) pair lies in this gap and causes a sharp change in direction. The region beyond this area is not structurally well defined, but 17 residues from the G there is a sequence, (aromatic)XX(P,D), that is very helpful in making alignments. It is followed four to five residues later by the PERF sequence, with some variations, as in PSRF, PDNF, PQRW, and so on. The PERF motif is not present in bacterial B-like P450 sequences, although it is in CYP102, CYP110, and CYP118 bacterial E-like P450s. It is part of the meander before the heme thiolate ligand (nomenclature of Peterson and Graham-Lorence; *see* **ref. *11***). After this point, there are length variations before reaching the signature sequence of all P450s, FXXGXXXCXG, with some variations allowed except at Cys. Even this one invariant residue has been replaced by His in two sequences from *Ciona intestinalis* (Genbank AK173774) and *Ciona savignyi*. Beyond the signature, 18 residues from the Cys, there is a conserved tetrapeptide, LQNF, or variants of it. The C-terminal region is quite variable, with an unexplained occurrence of a PR approx 33–34 residues from the F in LQNF. In many

**Table 1**
**Percentage of Identity Among Some Orthologs in *Drosophila, Anopheles,* and *Apis*[a]**

| | *Drosophila melanogaster* | *Drosophila pseudoobscura* | *Anopheles gambiae* | *Apis mellifera* |
|---|---|---|---|---|
| *18A1* | 100 | 96 | Absent | 60 |
| *49A1* | 100 | 91 | 63 | 46 |
| *301A1* | 100 | 89 | 75 | 69 |
| *302A1 dib* | 100 | 82 | 56 | 48[b] |
| *303A1* | 100 | 89 | 49 | 43[b] |
| *306A1 phm* | 100 | 66 | 48 | 47 |
| *314A1 shd* | 100 | 90 | 49 | 45 |
| *315A1 sad* | 100 | 79 | 36 | 38 |

[a]Values are from a comparison to *Drosophila melanogaster*. *dib, phm, shd,* and *sad* are Halloween genes important in development.

[b]Incomplete *Apis* sequences.

sequences, these are the last two amino acid residues, whereas in others there is an extension beyond the PR sequence.

These motifs form the main alignment benchmarks that are used in making P450 sequence alignments. Often the distance between benchmarks is absolutely the same, and a cursory check for reasonable homologies between the segments can confirm that the alignment is probably correct without introducing gaps. Of course, this is most difficult to do in the N-terminal region and when aligning bacterial or lower eukaryotic sequences that are very divergent from the other sequences.

## 4. Orthologs and Nomenclature

The world of P450 genes has expanded well beyond the limited mammalian realm. Because mammals dominated the early P450 studies, the rules for naming P450s were based largely on P450 statistics from mammals. Orthologs in mammals tend to be very conserved, ranging from 66% for *CYP17A1* to 96% for *CYP26B1* for mouse and human. The average is about 81%. These values fit nicely with the 40% rule for family membership and the 55% rule for subfamily membership. However, when more diverse phyla are considered, such as Arthropoda, the orthologs are not as strongly conserved. **Table 1** shows that orthologs in the single genus *Drosophila* are about as divergent as the orthologs between mouse and human. Orthologs among *Drosophila, Anopheles,* and *Apis* (honeybee) fall outside the 55% rule frequently and they even fall outside the 40% rule for *CYP315A1*. The *Anopheles* and *Apis 315A1* sequences have very low identities to *Drosophila 315A1*; however, the best BLAST scores for these sequences are clearly to *CYP315A1*. Niwa et al. *(5)* biochemically identified silkworm *315A1* as the ortholog of the *Drosophila* shadow (sad) gene, yet it only has a 36% identity to the *Drosophila* sequence, so the low percentage identity is not owing to mislabeling the orthologs. The value of the nomenclature is diminished if orthologs are placed in different subfamilies and even different families. When possible,

orthologs are given the same name, even if it violates the 55% rule or even the 40% rule.

## 5. Pseudogenes and Nomenclature

Pseudogenes are abundant in humans and mice and less common in fungi or bacteria. These faulty genes pose a nomenclature problem ***(12)***. They were originally tagged with a P on the end of the CYP name to indicate a pseudogene, as in *CYP2T2P*. As whole genomes were sequenced, it became apparent that there were at least four classes of pseudogene. The nearly intact pseudogene is still tagged with a P. The solo exon pseudogene is a small piece of a gene, just one or a few exons, far away from any other intact P450 genes. These have been named with an extension on the CYP name, as in *CYP4F-se1[6:7]*, in which -se1 stands for solo exon one of the CYP4F subfamily. The [6:7] indicates which exons of the *CYP4F* gene are present in the pseudogene. Detritus exons are indicted by the extension -de. These pseudogenes are close to a known gene and represent recent duplications of one or more exons from that gene. These exons may be alternative splice exons. The fourth type of pseudogene is an internal exon duplication. These pseudogenes may be intact or partial exons that occur inside a known gene. These exons form alternative transcripts. They are named with the extension -ie, for internal exon. The exact details of pseudogene naming are complicated. These are described in an article on all human and mouse P450 genes ***(13)***. Also in that article is a nomenclature for naming P450 alternative transcripts.

## 6. P450 Clans

The nomenclature for P450s used to be small, with only a few dozen families. Taxa such as vertebrates had fewer than 20 families, and one could easily memorize the family names. More diverse groups such as plants now have 62 families, and this is slowly growing. Fungi and bacteria are so diverse that every second or third sequence is in a new family. One cannot memorize the family names and recognize relationships between sequences in that way with these highly diverse groups. To restore some order to this exploding nomenclature system, higher-order groups were proposed. These are called clans, and they are essentially like clades, although clades technically refer to species with a common ancestor and not to sequences.

Clans have been defined as groups of P450 families that consistently cluster together on phylogenetic trees. No percentage of identity cutoff is given, because that would not work in this case. The 62 families of plant P450s sort into just 10 clans, once again a number that is reasonable and can be grasped and remembered. For details on plant P450 clans, *see* the article by Nelson et al. ***(8)*** on comparative genomics of rice and *Arabidopsis* P450s. The animal clans are still being defined, but it is clear that the insect CYP6 and CYP9 families belong in a clan with vertebrate CYP3 and CYP5. This has been called the CYP3 clan for the lowest family number in the group. Insects appear to have four clans, CYP2, CYP3, CYP4, and mitochondrial. These clans have families that cluster with vertebrate CYP2, CYP3, CYP4, and mitochondrial (CYP11, CYP24, CYP27) families. Vertebrates have about 10 clans, although some, such as CYP19, have only one family. The clans that are in common between insects and

vertebrates must have had a common ancestral sequence in the bilaterian ancestor of animals. For a tree with vertebrate P450 clans shown, see my article on the comparison of human and Fugu P450s ***(14)***. There is no comprehensive assignment of vertebrate and invertebrate clans yet.

## 7. Of CYPs and CYPs

The CYP root of cytochrome P450 gene names is not exclusively used for P450 genes. Without realizing that this root term was used for cytochrome P450, it has also been used for cyclophilins. This has happened in *Caenorhabditis elegans*, mammals, *Arabidopsis,* and *Chlamydomonas.* The Committee on Standardized Cytochrome P450 Nomenclature has been trying to correct this ***(15)***. The HUGO Human and Mouse Gene Nomenclature Committees recently approved the use of *CYN* in human and *Cyn* in mouse for the official gene names for cyclophilins. The *C. elegans* community also agreed to change 17 official names from *CYP* to *CYN*. Unfortunately, in the issue of *Plant Physiology* preceding the one that carried the official nomenclature for 455 rice *CYP* genes ***(8),*** an article was published naming 29 *Arabidopsis* cyclophilins as *CYP* genes ***(16)***. This has not yet been addressed. Only two cyclophilin genes in *Chlamydomonas* had been named *CYP*, and it has been agreed to change them to *CYN*. These types of nomenclature problems are long lasting because they are published in the literature and are perpetuated in databases. It may take many years to clear up this confusion.

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2

# Spectral Analyses of Cytochromes P450

**John B. Schenkman and Ingela Jansson**

**Summary**

UV/Vis spectroscopy is the major means of identifying intact holocytochrome P450. The carbon monoxide complex of the intact ferrous hemoprotein exhibits a characteristic spectrum between 448 and 452 nm, considerably distinct from the usual Soret absorption peaks of hemoproteins. Methods are described for identification and quantitation of cytochrome P450 (CYP) in membranes, in tissue homogenates, and in purified form, using difference spectroscopy and absolute spectroscopy. CYP are b-type cytochromes, containing protoporphyrin IX as the prosthetic group. Methods are also provided, using alkali and pyridine, for quantitation of the hemoprotein by this prosthetic group. In its oxidized, or ferric state, CYP exists as an equilibrium mixture of high- and low-spin configurations, each with distinctive UV/Vis absorption peaks. Substrate binding causes shifts in the spin equilibrium, and methods are shown for using these shifts for quantitation of substrate binding to CYP.

**Key Words:** UV/Vis; difference spectrum; absolute spectrum; cytochrome P450 (CYP) spectrum; hemochromogen; pyridine hemochromogen; binding spectrum; dithionite; carbon monoxide complex.

## 1. Introduction

Cytochrome P450 (CYP) was first shown to be a hemoprotein in 1964 *(1)*. It has as its prosthetic group a noncovalently bound iron protoporphyrin IX, a form of heme found in b-type cytochromes, such as cytochrome-*b*, hemoglobin, and myoglobin. This type of heme prosthetic group can be removed from the protein by acidic acetone or by alkali and pyridine. A number of methods can be used for spectrophotometric analysis of hemoproteins, each useful for a different purpose. In this chapter, we provide methods for quantifying CYP, as well as methods for studying substrate interaction with the purified protein, the hemoprotein in membranes, and in tissue homogenates. The ability of those hemoproteins with an available sixth coordination position to bind small molecules such as cyanide, carbon monoxide (CO), and ethyl isocyanide has

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provided means for their analysis and quantification. In the case of CYP, the binding of CO permitted demonstration of its involvement in the monooxygenase reaction.

## 2. Materials

### 2.1. Determination of Heme

1. 0.4 *M* NaOH containing 40% pyridine.

### 2.2. Determination of Hemoprotein

1. Buffered sucrose for homogenate: 0.25 *M* sucrose, 10 m*M* Tris-HCl, pH 7.25.
2. Buffer for microsomes: 50 m*M* Tris-HCl, pH 7.25.
3. Buffer for purified cytochrome P450: 50 m*M* sodium phosphate, pH 7.25; 20% glycerol.
4. Oxygen-free CO tank with an appropriate two-stage valve to permit very slow bubbling of the gas into CYP-containing solutions.
5. Dilauroylphosphatidylcholine: 8 m*M* (5 mg/mL) in 50 m*M* sodium phosphate, pH 7.25; 20% glycerol; 0.1 *M* NaCl; 5 m*M* ethylenediaminetetra-acetic acid (EDTA). This is best prepared fresh and dispersed in a bath sonifier until the mixture clarifies to a mild opalescence.
6. Dual-beam spectrophotometer or machine capable of yielding a difference spectrum.
7. Dithionite (sodium hydrosulfite, $Na_2S_2O_4$).
8. 0.5 m*M* dithionite solution. This must be prepared fresh and is best accomplished by adding dry sodium dithionite to a small rubber-stoppered vial, gassing with argon or nitrogen through needles to remove oxygen, and then injecting degassed buffer into the vial to dissolve the dithionite.

### 2.3. Substrate Binding

1. Microsomes or purified CYP at a concentration of 1 μ*M* CYP or higher in buffer as described in **Subheading 2.2.**
2. Substrates of CYP, 100 m*M* in water: ethylbenzene, hexobarbital, aminopyrine, ethylmorphine, or any of a wide variety of drugs or organic chemical compounds.

## 3. Methods

### 3.1. Quantification of Heme and Hemoprotein

#### 3.1.1. Pyridine Hemochromogen (see **Notes 1–4**)

CYP, like many b-type hemoproteins, contains a single-heme prosthetic group that can be displaced and complexed with alkaline pyridine. This provides a means to quantify the enzyme when it is not possible to do so by other methods. Interestingly, it was the ability of this method to quantify heme in liver microsomes that provided the first clue that a hemoprotein other than cytochrome $b_5$ was present in the microsomes.

1. Dilute a sample of microsomes or purified CYP to 2 mL in a final concentration of 0.2 *M* NaOH, 20% pyridine (*see* **Note 1**).
2. Divide the sample into two cuvets, and record the baseline of equal light absorption between them from 600 to 500 nm in a dual-beam spectrophotometer (*see* **Note 2**).
3. Add a few milligrams of sodium dithionite on the tip of a spatula only to the sample cuvet and record a spectrum from 600 to 500 nm. The difference in light absorption changes at 557 nm relative to 575 nm ($\Delta A_{557-575}$) is read directly from the recording (*see* **Fig. 1**).

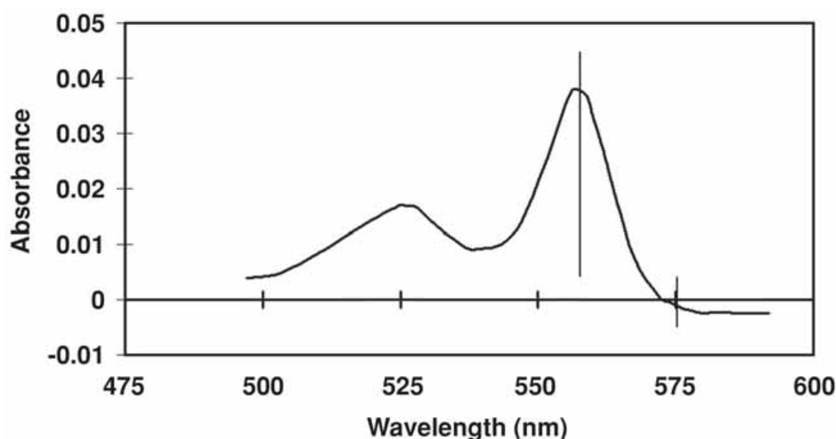


Fig. 1. Pyridine hemocromogen. Sample dissolved in 20% pyridine in 0.2 *M* NaOH. Vertical lines indicate 557 nm (long line) and 575 nm have a ΔA of 0.041 and indicate a heme content of 1.27 µ*M*.

4. Calculate the heme concentration (*c*) using Beer's Law: $A = \varepsilon \cdot c \cdot L$. *A* would be the difference in light absorption changes at 557 nm relative to 575 nm. The light path (*l*) of the cuvet is 1 cm, and the difference extinction coefficient ($\varepsilon_{557-575}$) for iron protoporphyrin (heme) is 32.4 m$M^{-1}$ cm$^{-1}$ ***(1,2)*** (*see* **Note 3**).
5. If the hemoprotein has been purified and its protein content has been measured, determine the extent of purity by expressing the data in terms of nanomoles of heme/miiligram of protein (*see* **Note 4**).

### 3.1.2. CO Complex (see *Notes 5–8*)

#### 3.1.2.1. Cytochrome P450 in Microsomes

1. Suspend an aliquot of a microsomal preparation (or purified preparation; *see* **Fig. 2**) in 2 mL of buffer (*see* **Note 5**) and divide it into two cuvets.
2. Record a baseline spectrum of equal light absorption between the cuvets in a dual-beam recording spectrophotometer between 500 and 400 nm.
3. Bubble CO slowly into the sample cuvet for about 30 s (*see* **Note 6**).
4. Add a few milligrams of sodium dithionite on the tip of a spatula to the sample cuvet, stir, wait about 1 min, and then record the difference spectrum (*see* **Note 7** and **Fig. 2**).
5. Calculate the concentration of CYP in the cuvet from the absorption change at 450 nm relative to the absorbance change at 490 nm using the Beer's Law equation in **Subheading 3.1.1., step 4** and the extinction coefficient $\varepsilon_{450-490}$ = 91 m$M^{-1}$ cm$^{-1}$ ***(1)***.

#### 3.1.2.2. Cytochrome P450 Spectrum in Tissue Homogenates

Because there are a number of different pigments in cells, including mitochondrial flavoproteins and hemoproteins, plus cytoplasmic pigments, such as the endoplasmic reticulum cytochrome-$b_5$, caution must be exercised in attempting to quantify CYP in whole homogenates. The methods described next allow one to obviate the influences

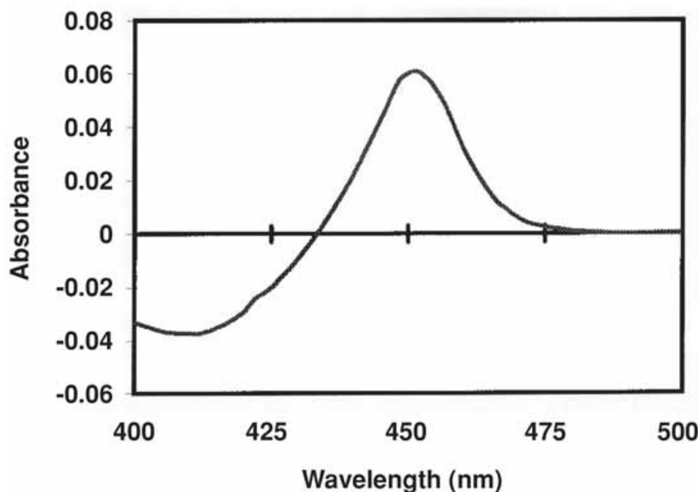


Fig. 2. Carbon monoxide difference spectrum of purified cytochrome P450 2B4 in 50 m*M* sodium phosphate, pH 7.25, 20% glycerol and 0.2% Emulgen 911. The absorbance difference between 450 nm and 490 nm is 0.062, indicating a concentration of 0.68 μ*M*.

of such pigments on the CYP spectrum in homogenates, or mitochondrial preparations, and even allow monitoring of this pigment in tissue slices ***(3)***.

1. Prepare a 1:10 (g/mL) homogenate of liver in sucrose buffer.
2. Transfer one aliquot into each of two cuvets, and record a baseline of equal light absorption in a dual-beam spectrophotometer between 500 and 400 nm.
3. Bubble nitrogen into the reference cuvet and CO into the sample cuvet, each for 30 s.
4. Stir an equal amount (10 μL) of dithionite solution into each cuvet and record the spectrum (*see* **Note 8**).

## 3.2. Hemoprotein Spectra

### 3.2.1. Absolute Spectra of Cytochrome P450 (see **Notes 9–11**)

#### 3.2.1.1. Ferric Cytochrome P450

1. With purified hemoprotein, dilute to a concentration of about 1 μ*M* in 50 m*M* sodium phosphate buffer, pH 7.25, containing 20% glycerol (*see* **Note 9**).
2. Fill two cuvets with the buffer and record a baseline of light absorption of the buffer in a dual-beam spectrophotometer from 670 to 250 nm.
3. Replace the buffer in the sample cuvet with hemoprotein in the same buffer and record the spectrum (*see* **Note 10**). A spectrum of CYP2B4 is shown in **Fig. 3**.

#### 3.2.1.2. Ferrous Cytochrome P450

1. Fill two anaerobic cuvets with 50 n*M* sodium phosphate buffer, pH 7.5, containing 20% glycerol, and record a baseline of equal light absorption of a buffer in dual-beam spectrophotometer from 670 to 300 nm.
2. Replace the buffer in the sample cuvet with hemoprotein in the same buffer, degas the cuvet to remove oxygen, and record the ferric hemoprotein absolute spectrum.

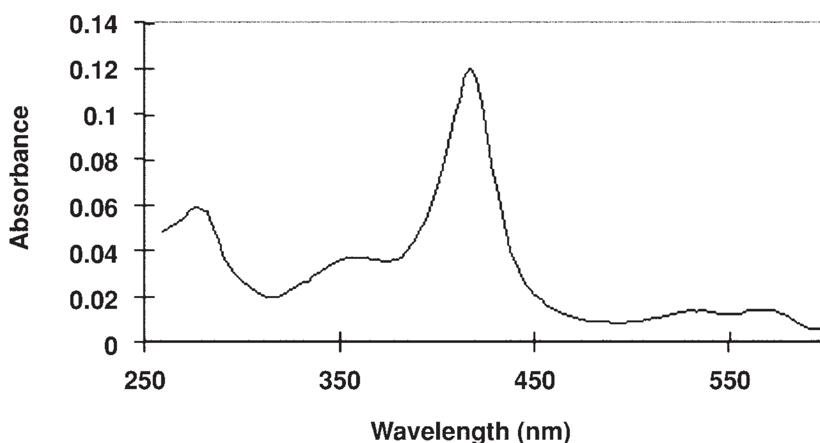


Fig. 3. Absolute spectrum of 0.96 µ*M* CYP2B4 in 20% glycerol, 50 m*M* sodium phosphate, pH 7.25. In the absence of substrate and at room temperature this approx 95% low spin. The charge transfer band at 650 nm (not shown) is not detectable at this concentration of cytochrome P450.

3. Inject an amount (a few microliters) of dithionite solution sufficient to reduce the CYP (equimolar) to each cuvet (*see* **Note 11**), and record the spectrum at 2-min intervals until no further reduction is seen. Add an additional injection of dithionite to ensure full reduction.

#### 3.2.1.3. CO Complex of Cytochrome P450

1. Fill two anaerobic cuvets with the buffer and record a baseline of equal light absorption of the buffer in a dual-beam spectrophotometer from 670 to 300 nm.
2. Replace the buffer in the sample cuvet with hemoprotein in the same buffer.
3. Degas the cuvet and replace the gas phase with oxygen-free CO.
4. Inject an amount (a few microliters) of dithionite solution sufficient to reduce the hemoprotein (equimolar) to each cuvet, and record spectra at 2-min intervals until no further reduction (*see* **Note 11**). Add an additional injection of dithionite to ensure full reduction.

### *3.2.2. Substrate-Binding Difference Spectra (see* ***Notes 12–15****)*

Ferric CYP interacts with a number of its substrates with a concomitant shift in the equilibrium between the high-spin (390-nm absorption maximum) and low-spin (417-nm absorption maximum) configurations. The spectral changes seen are small compared with the absolute spectral absorption and are best monitored in difference spectra (*see* **Note 12** and **Fig. 4**).

1. Prepare a suspension of microsomes in buffer, or purified hemoprotein reconstituted with phospholipid in glycerol-containing buffer, with a CYP concentration of at least 1 µ*M* in buffer. If purified CYP is used, add it to the sonicated phospholipid at a molar ratio of P450 to lipid of 1:160 and allow it to interact at room temperature for a least 30 min (*see* **Note 13**).
2. Record a baseline of equal light absorption in the region of study (usually 450–350 nm).

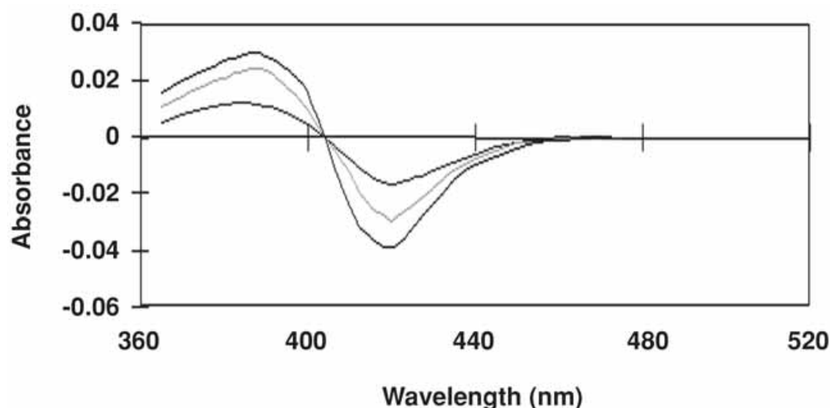


Fig. 4. Substrate induced difference spectra. Titration of spectral changes induced by addition of increasing concentrations of hexobarbital to rat liver microsomes and equal volumes of vehicle to the reference cuvet. Concentrations were 0.08 m*M*, 0.33 m*M* and 0.66 m*M*, respectively.

3. Add 0.1–1 m*M* concentrated substrate solution, and record a difference spectrum after each addition. The total volume of the additions should not exceed 2% of the volume in the cuvet (*see* **Notes 14** and **15**). Add an equal volume of vehicle to the reference cuvet.

## 4. Notes

1. Smaller or larger volumes can be used as needed.
2. Set the sensitivity of the machine (absorbance range) appropriately. For example, a 1-nmol sample of hemoprotein in 1 mL would have a $\Delta A$ of 0.0324.
   a. One may also record absolute spectra, instead of difference spectra, thereby using half the amount of material. First, record a baseline with alkaline pyridine in each of two cuvets. Place a sample in one cuvet and record the absolute spectrum of the ferric pyridine complex vs the alkaline pyridine. Add a few milligrams of dithionite to the sample- and solvent-containing cuvets and record the spectrum. Draw vertical lines between the oxidized and dithionite-reduced spectra at 557 and 575 nm. Subtract the differences in absorbance between the two wavelengths. This is the $\Delta A_{557-575}$.
   b. One can also do a pyridine hemochrome measurement without a dual-beam spectrophotometer, e.g., with a Gilford spectrophotometer, using one cuvet at a time: prepare enough sample in NaOH and pyridine for two cuvets. Zero the light absorption of the sample in the first cuvet at 575 nm, add a few milligrams of sodium dithionite, and record the absorbance change at 575 nm. Repeat the procedure with the other cuvet at 557 nm. Subtract the absorbance change ($\Delta A$) at 575 nm from that at 557 nm. This will give the $\Delta A_{557-575}$.
3. *A* is really $\Delta A$, the absorbance difference of a wavelength couple. If the difference in absorbance changes at 557 nm relative to 575 nm was 0.1, then the heme concentration of the mixture would be 0.0031 m*M*, or 3.1 nmol/mL.
4. With purified CYP forms, the specific content values obtained are between 17 and 20 nmol/mg of protein.
5. If purified CYP is to be used, a detergent (e.g., 0.2% Emulgen 911 or Triton N101) is added to diminish aggregation, unless phospholipid (e.g., dilauroylphosphatidylcholine,

160:1 molar ratio) is present, and 20% glycerol is included to stabilize the hemoprotein from conversion into P420, a breakdown product. For kinetic measurements of CYP reduction, one can monitor the absorption changes at 450 nm relative to the isosbestic wavelength of 490 nm.

6. The dissolved CO will reach a concentration of 1 m*M*. It is best to bubble CO into the sample before adding the chemical reductant, because lower values are obtained if the sequence is reversed. The ferrous hemoprotein is somewhat unstable and is destroyed, perhaps by hydrogen peroxide generated by reduction of oxygen during the bubbling.
7. The reduction of CYP is not a fast reaction, even with the strong reductant sodium dithionite, hence the need to wait at least 1 min. It is often useful to wait an additional minute and repeat the scan to ensure that reduction has gone to completion. It is possible, if desired, to measure the amount of cytochrome-$b_5$ in microsomes prior to quantifying the CYP content. To do this, add reduced nicotinamide adenine dinucleotide (to a final concentration of 0.1 m*M*) just to the sample cuvet before the addition of dithionite. This will rapidly and completely reduce cytochrome-$b_5$ (only minimally reducing CYP), yielding a reduced minus oxidized spectrum. To quantify the concentration of this hemoprotein use $\varepsilon_{424-490} = 112\ \mathrm{m}M^{-1}\ \mathrm{cm}^{-1}$ (**4**).
8. By reducing both cuvets, the contribution of microsomal cytochrome-$b_5$ and all of the mitochondrial pigments except cytochrome oxidase is canceled out. The concentration of CYP in liver tissue is about five times that of any individual mitochondrial pigment, and the difference spectrum of the CO complex of cytochrome oxidase has a minimal impact on it.

   However, if the concentration of cytochrome P450 is low relative to the other cellular CO-binding pigments, such as cytochrome oxidase in the mitochondria, it may be necessary to cancel such pigments with another ligand, such as cyanide. Cyanide binds tightly to cytochrome oxidase but has a low affinity for CYP (the dissociation constant is 2.1 m*M*); hence, the addition of 1 m*M* potassium cyanide to both cuvets before bubbling in CO will diminish the contribution of cytochrome oxidase to the difference spectrum of CYP.
9. The Soret extinction coefficient of most hemoproteins is in the range of $100\ \mathrm{m}M^{-1}\ \mathrm{cm}^{-1}$, so to obtain a good spectrum, one must choose a suitable hemoprotein concentration and an appropriate sensitivity setting. For example, if the concentration of CYP was 1 μ*M*, an absorption of about 0.1 for the Soret peak would be expected. It is best not to add a nonionic detergent because its absorption in the UV will obliterate the spectrum below 400 nm. An absorbance ratio of $A_{417}/A_{280}$ between 1 and 2 is a good indicator of purity of the hemoprotein, with the 280-nm absorption owing to the aromatic amino acids, and the 417-nm absorption to the heme complex. The hemoproteins will have different molar amounts of aromatic residues, and the spin equilibrium will, predictably, influence the ratio.
10. The ferric hemoprotein has a small 650-nm charge-transfer absorption band attributable to the high-spin configuration, and α- and β-absorption bands at 570 and 535 nm, respectively, in the low-spin configuration. Its Soret (γ-band) is at 417 nm and is typical of a low-spin state, but it is in equilibrium with a variable proportion of high-spin configuration that has an absorption peak at about 395 nm. The proportion of high-spin hemoprotein can be increased by adding certain substrates or by elevating the temperature of the solution, because the spin equilibrium is also temperature dependent.
11. Sodium dithionite strongly absorbs in the UV; hence, it is advisable to use the minimal amount that will reduce the CYP and provide an equal amount to the reference cuvet. For example, if the CYP concentration is 1 μ*M*, the addition of 2 μL of dithionite solution

would be just sufficient to reduce the hemoprotein without causing a perturbation of the absolute spectrum.

12. The magnitude of the absorption change induced by substrate is rather small, generally <20% of the overall absorption of the membranous CYP in the Soret region, hence the need for sufficient amount of hemoprotein or a spectrophotometer with sufficient sensitivity and ability to monitor the spectral changes in the difference spectrum. Bacterial cytochrome $P450_{cam}$ (CYP101) undergoes a full shift in its low-spin peak, from 417 to 390 nm (high spin), on addition of its substrate, camphor.
13. Purified mammalian CYPs tend to aggregate, and in the absence of phospholipid it is generally not possible to obtain substrate-induced shifts in the spin equilibrium.
14. Because the spectral changes are relatively small, the addition of larger volumes of liquid to the cuvet can result in minor dilution differences between the cuvets that can give rise to an erroneous difference spectrum. For example, with a concentration of CYP of 1 μ*M*, an imbalance in the dilution of 1% could cause a spectral imbalance of 0.001 absorbance, a value on the order of magnitude of changes invoked by the addition of some substrates.
15. Some substrates are poorly water soluble. If added in another solvent, such as ethanol, be aware that many organic solvents are able to perturb the spectrum of CYP. Although these may be blanked out by the addition of a similar amount of solvent to the reference cuvet, such additions can alter data and calculations such as that of a dissociation constant. Substrate concentrations generating maximal spectral changes with different substrates range from 0.1 to 1 m*M*.

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# 3

## Establishment of Functional Human Cytochrome P450 Monooxygenase Systems in *Escherichia coli*

**Michael P. Pritchard, Lesley McLaughlin, and Thomas Friedberg**

**Summary**

Cytochromes P450 (CYP) have been expressed in a variety of systems such as mammalian cells, yeast, and bacteria. The bacterial system is technically the least demanding and provides large amounts of catalytically active P450s for metabolic and structural studies relating to preclinical drug development. This chapter provides a detailed technical description of the processes that allow the coexpression of various CYP isoforms together with CYP reductase in *Escherichia coli* and gives some examples of the results that can be achieved for the expression of human P450s.

**Key Words:** Cytochrome P450; cytochrome P450 oxidoreductase; bacterial expression systems; cDNAs; heterologous expression; preclinical drug development; drug metabolism

### 1. Introduction

An important task of preclinical drug development is to predict the metabolism of novel compounds in humans. Often this assessment relies on assays carried out in animals or with animal-derived tissues. Besides being often considered ethically and economically problematic, animal-based systems have limitations, partially owing to large species differences in the enzymes involved in drug metabolism ***(1)***. It is therefore essential to develop systems that are based on material derived from human tissues. These should significantly shorten the period between the discovery of a drug and its introduction into the market. Because cytochrome P450- (P450) mediated reactions are thought to catalyze the most important metabolic steps in the disposition of many drugs, recombinant cellular models for the various human P450 isoforms are an ideal tool to predict involvement of P450s in drug disposition in humans. We have developed these models in mammalian cells, in yeast, and *Escherichia coli* ***(2–5)***. From our experience and that of our collaborators in the pharmaceutical industry ***(6,7)***, the

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establishment, handling, and reproducibility of the bacterial expression systems is far superior to those of the former two cellular P450 models and are described herein.

## 2. Materials

### 2.1. Construction of Expression Plasmid(s)

1. pCWOri+ expression vector *(8)* for P450s.
2. pACYC-184 expression vector (New England Biolabs) for P450 reductase.
3. *E. coli* strains DH5α (Invitrogen, Carlsbad, CA) and JM109 (Promega, Madison, WI) (*see* **Note 1**).
4. Oligonucleotide primers to isolate P450 and P450 reductase cDNAs and to modify the cDNAs for bacterial expression.
5. Restriction enzymes, T4 DNA ligase, *Pfu* polymerase (Stratagene), and buffers.
6. Other general supplies for molecular biology work.

### 2.2. Coexpression of P450 and P450 Reductase in E. coli

1. Luria Bertani (LB) medium: dissolve 10 g/L of NaCl, 10 g/L of peptone, and 5 g/L of yeast extract in water and autoclave.
2. Modified Terrific Broth (TB): dissolve 12 g of bacto-tryptone, 24 g of bacto-yeast extract, and 2 g of bacto-peptone (all Difco, Detroit, MI) together with 4 mL of glycerol in 900 mL of water and sterilize by autoclaving. Just before use, add 100 mL of a sterile 10X phosphate solution consisting of 0.17 *M* $KH_2PO_4$ and 0.72 *M* $K_2HPO_4$.
3. Trace elements solution (X4000 stock): add about 50 mL of water to 2.45 g of ferric citrate in a beaker, stir over moderate heat until dissolved, and then leave to cool. Add 10 mL of concentrated HCl; the solution should turn straw yellow. Add 0.131 g of $ZnCl_2$, 0.2 g of $CoCl_2{\cdot}6H_2O$, 0.2 g of $Na_2MoO_4{\cdot}2H_2O$, 0.1 g of $CaCl_2{\cdot}2H_2O$, 0.127 g of $CuCl_2{\cdot}2H_2O$, and 0.05 g of $H_3BO_3$ and stir until dissolved. Make up to 100 mL with water, and then filter-sterilize (0.22 μ*M*). Store at room temperature.
4. 1 *M* Thiamine hydrochloride in water (filter-sterilized): store in aliquots at –20°C.
5. Ampicillin (50 mg/mL in water, filter-sterilized): store in aliquots at –20°C.
6. Chloramphenicol (25 mg/mL in ethanol): store in aliquots at –20°C.
7. 500 m*M* Isopropyl-β-D-thiogalactopyranoside (IPTG) in water (filter-sterilized): store in aliquots at –20°C.
8. 1 *M* δ-Aminolevulinic acid (ALA) in water (filter-sterilized): store in aliquots at –70°C.
9. Autoclave, conical flasks, shaking incubator.

### 2.3. Harvesting of Cells, Preparation of Spheroplasts, and Isolation of Membrane Fractions

1. 2X TSE buffer: 100 m*M* Tris-acetate, pH 7.6, containing 500 m*M* sucrose and 0.5 m*M* EDTA. Filter sterilize and store at 4°C.
2. Lysozyme (20 mg/mL in water, freshly prepared and kept on ice).
3. Spheroplast resuspension buffer: 100 m*M* potassium phosphate, pH 7.6, containing 6 m*M* magnesium acetate, 20% (v/v) glycerol, and 0.1 m*M* dithiothreitol (DTT) (filter-sterilized). Store complete solution in aliquots at –20°C, or prepare solution without DTT and store at room temperature, and add DDT from a separate 1 *M* stock (stored in aliquots at –20°C) just before use.
4. Protease inhibitors: 100 m*M* phenylmethylsulfonyl fluoride (PMSF), in isopropanol (X100 stock); 10 mg/mL of aprotinin, in 10 m*M* HEPES, pH 8.0 (X10,000 stock); 10 mg/mL

leupeptin, in water (X10,000 stock). Stock solutions of protease inhibitors are stored at –20°C. PMSF stock solution requires warming to room temperature and redissolving before use. Aprotinin stock solution is stored in small aliquots, because it must not be refrozen after thawing.

5. 1X TSE buffer: 50 m*M* Tris-acetate, pH 7.6, containing 250 m*M* sucrose and 0.25 m*M* EDTA. Filter-sterilize and store at 4°C.
6. Centrifuges and centrifuge tubes.
7. Dounce tissue grinder.

### 2.4. Analytical Procedures for Determination of Cytochrome P450 and Cytochrome P450 Reductase

1. P450 spectrum buffer (2X stock): 200 m*M* Tris-HCl, pH 7.4, containing 20 m*M* CHAPS, 40% (v/v) glycerol, and 2 m*M* EDTA. Filter-sterilize and store at 4°C.
2. Carbon monoxide (CO) cylinder in fume cupboard.
3. Sodium dithionite.
4. 50 μ*M* cytochrome-*c* in 0.3 *M* potassium phosphate buffer, pH 7.7.
5. 5 m*M* NADPH in water: prepare fresh and keep on ice.
6. Dual-beam spectrophotometer with attachment for turbid samples and cuvet heater.
7. Cuvets.

## 3. Methods

The generation of a functional P450 monooxygenase system in *E. coli* requires the coexpression of P450s together with the ancillary protein P450 nicotinamide adenine dinucleotide phosphate (NADPH) oxido reductase (P450 reductase), which supplies P450s with electrons from NADPH. The methods outlined describe: (1) the isolation of P450 and P450 reductase cDNAs, their modification for bacterial expression, and their ligation into the expression plasmid; (2) the technology for the expression of human P450 cDNAs in *E. coli*; and (3) the isolation of membrane fractions containing the expressed recombinant P450s.

### 3.1. Isolation of P450 cDNA and Cloning Into Expression Plasmid pCWOri+

The methods outlined in this chapter are based on standard methods of genetic engineering and only the general cloning strategies are described. The coding regions of P450 cDNAs can be isolated by reverse transcriptase-polymerase chain reaction (RT-PCR) employing primers that are designed based on the sequence information available in gene databases. Hepatic mRNA is used as template unless extrahepatic P450s need to be isolated. *Pfu* polymerase is employed in the second-strand synthesis to reduce the incidence of replication errors. The subcloned bonafide cDNAs serve as templates for the subsequent reactions. To allow functional expression in *E. coli*, the N-terminal coding region of mammalian P450 cDNAs requires modification. We have developed a modification strategy (*ompA*+2 strategy), whereby a cDNA fragment encoding the bacterial *ompA* leader sequence (21 amino acid residues) and two additional spacer amino acid residues (Ala-Pro) are fused to the P450 cDNA in frame with the P450 initiation codon (*see* **Note 2**). Compared with other strategies (17-α strategy) employed for the expression of P450s in *E. coli* ***(9,10)***, the *ompA*+2 strategy has the advantage that it does not require changes within the coding region of P450 cDNAs. In addition, the leader

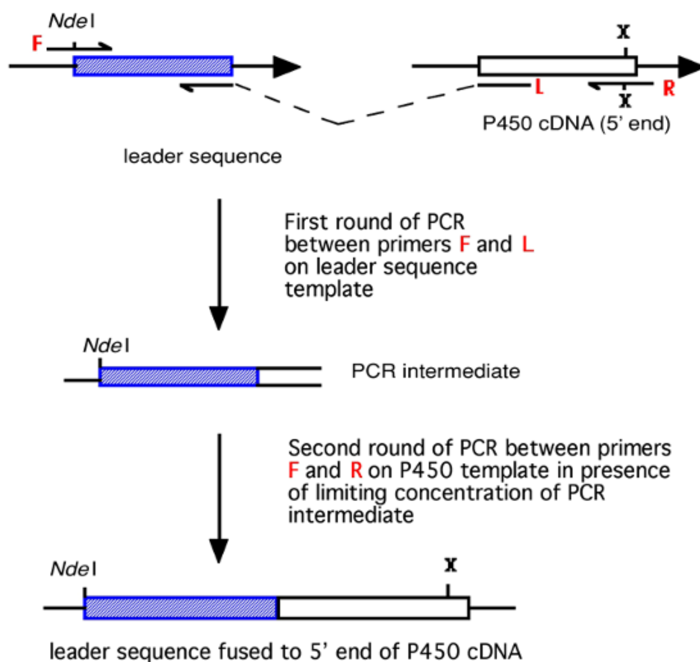



Fig. 1. Details of primers used are given in **Table 1**. The first round of PCR uses a leader sequence-specific forward primer (F) and a reverse "linker" primer (L) complementary to at least the first 21 bases of the P450 cDNA joined to the last 21 bases of the leader sequence, thus specifying the join. Suggested DNA templates are the plasmid pET-20b and bacterial genomic DNA from *E. coli* strain JM109, for the *pelB* and *ompA* leaders, respectively. The resulting intermediate PCR product is purified and then used, at limiting concentration, in a second round of PCR using the same forward primer (F) and a reverse primer (R) complementary to the P450 cDNA of interest, binding across or immediately 3' to a unique internal restriction enzyme site (X). The suggested DNA template in this case is a plasmid containing the P450 cDNA. Only after interstrand annealing between the PCR intermediate (which is quickly exhausted from the reaction) and the P450 cDNA template, followed by elongation and synthesis of the second strand (primed by primer R), can PCR occur to yield the desired product.

sequence is proteolytically removed during bacterial synthesis, thus releasing the native cytochrome P450. Similarly, P450 reductase is modified with a *pelB* leader to allow optimal functional bacterial expression of this ancillary protein. **Figure 1** exemplifies the general PCR-based method for merging the *ompA*+2 leader sequences to any P450 cDNA. Note that PCR is performed in such a way as to allow the incorporation of 5'and 3' restriction sites into the final PCR products, to facilitate cloning into expression vectors. The 5' restriction site in the forward primers is always *Nde*I, which incorporates the initiation codon and is the most 5' restriction site in the polylinker of the expression vector pCWOri+ (*see* **Fig. 2**). Even though the fusion method can, in principle, be used to isolate the full-length templates encoding the enzymes fused to leader sequences in

**Table 1**
**Sequences of Primers Employed for Fusion of Leader Sequences to Either P450s or P450 Reductase**[a]

| Designation | Sequence |
|---|---|
| *ompA*+2—forward | GGAATTCCATATGAAAAAGACAGCTATCGCG |
| *ompA*+2—linker | XXXXXCGGGACGGCCTGCGCTACGGTAGCGAA |
| *pelB*-reductase—forward | GGAGATATACATATGAAATACCTGC |
| *pelB*-reductase—linker | TCCACGTGGGAGTCTCCCATGGCCATCGCCGGCTGGGCAGC |

[a] XXXXX, sequence specific for each P450 isoform; sequence underlined in the *pelB*-reductase linker is complementary to the P450 reductase, whereas the remainder is complementary to parts of the *pelB* leader sequence. *See* **Fig. 1** for an explanation of the fusion strategy.

one step, it is advisable to merge first only a part of the 5' region of the cDNAs to the leader sequence. This is followed by ligation of the merged sequence to the remaining 3' end of the cDNAs in holding vectors via a unique restriction site, in order to reconstitute the full-length cDNA merged to the bacterial leader sequence.

**Table 1** displays the 5' forward primer sequences employed for fusing the *ompA*+2 leader to any P450 cDNA. The leader sequence, but not the P450-specific part of the linking primer, is also given. The sequence of the reverse primer is specific to the P450 cDNA and is not displayed. In the final expression construct, the stop codon is provided by the P450 cDNA.

The template encoding the P450 reductase fused to the *pelB* leader can also be isolated using the fusion strategy displayed in **Fig. 1**. **Table 1** displays the forward and the linking primer suitable for the fusion of the bacterial *pelB* leader to the P450 reductase cDNA by PCR.

**Figure 2** displays the N-terminal region of the P450 isoform CYP3A4 either fused to the *ompA*+2 leader or modified for expression using the 17-α strategy. In addition, **Fig. 2** displays the N-terminal sequence of the P450 reductase fused to the *pelB* leader sequence. The expression vector for P450 is pCWOri+ (conferring ampicillin resistance), and that of the P450 reductase is pACYC-184 (conferring resistance to chloramphenicol) (**Fig. 2**). Because the latter vector does not contain an IPTG-inducible promoter for the expression of the P450 reductase, this promoter must first be added to the *pelB*-reductase via intermediate subcloning into pCWOri+. This is best achieved by including an *Nde*I site at the 5' end of the *pelB*-reductase insert. The promoter and cDNA can then be released from this vector by a *Bcl*I-*Bgl*II double digest (*see* **Note 3**) and inserted into the *Bam*HI site of pACYC-184, thereby abolishing its tetracycline-resistance gene but maintaining the gene encoding resistance to chloramphenicol.

### *3.2. Coexpression of P450 and P450 Reductase in* E. coli

1. Prepare a starter culture of the bacterial strain of interest by inoculating 5–10 mL of LB broth containing ampicillin (50 µg/mL) and chloramphenicol (25 µg/mL) with a single, isolated colony picked from an LB-agar plate (supplemented with ampicillin and chloramphenicol as previously stated). Shake overnight at 37°C (200 rpm) (*see* **Notes 4** and **5**).

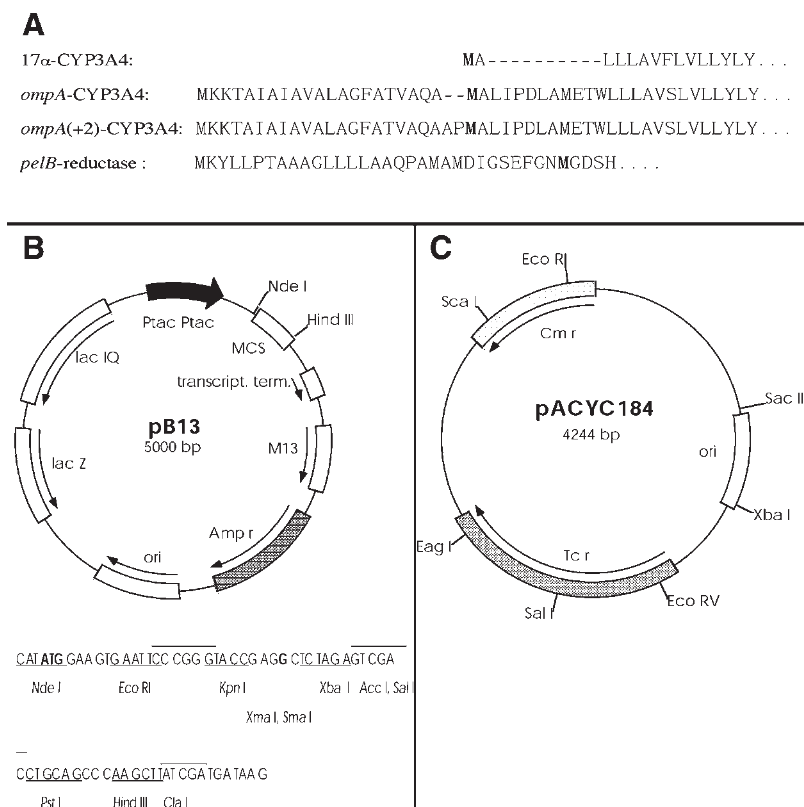
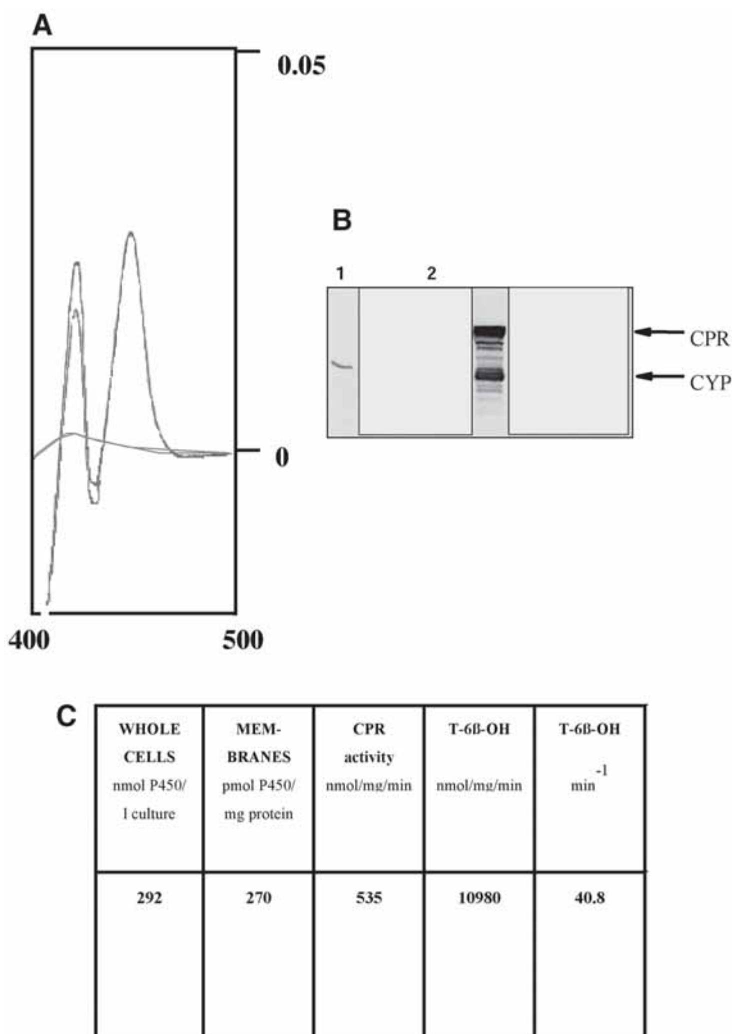



Fig. 2. **(A)** N-terminal amino acid sequences of CYP3A4 modified at its N-terminus using either the 17-α strategy or fusion to either the *ompA* or the *ompA*(+2) leader with the latter containing a spacer of two amino acid residues between the *ompA* leader and the P450. Note that the latter strategy increases the probability of the leader being proteolytically removed during biosynthesis, thus releasing the native P450. **(B)** The vector pCW (Ori+) (pB13) used for the expression of P450s and **(C)** the vector pACYC184 employed for the expression of P450 reductase are shown.

2. The next morning, dilute the overnight culture 1:100 into TB (*see* **Note 6**) supplemented with ampicillin (50 µg/mL), chloramphenicol (25 µg/mL), thiamine (1 m*M*), and trace elements (*see* **Note 7**) in a conical flask (*see* **Note 8**). Shake at 30°C and 200 rpm. After about 4 h, start monitoring the $OD_{600}$ of the culture at regular intervals (*see* **Note 9**).
3. When the $OD_{600}$ of the expression culture reaches 0.7–1.0, add ALA to 0.5 m*M* and IPTG to 1 m*M* (*see* **Notes 10–12**). Incubate at 30°C for a further 19–22 h or longer if required (*see* **Notes 13–15**).

### 3.3. Harvesting of Cells, Preparation of Spheroplasts, and Isolation of Membranes

1. Pour the cells into suitable centrifuge tubes or bottles and chill on ice for about 10 min. Pellet the cells by centrifuging at 2800*g* for 20 min at 4°C.




| WHOLE CELLS nmol P450/ l culture | MEM-BRANES pmol P450/ mg protein | CPR activity nmol/mg/min | T-6ß-OH nmol/mg/min | T-6ß-OH $min^{-1}$ |
|---|---|---|---|---|
| 292 | 270 | 535 | 10980 | 40.8 |

Fig. 3. Analysis of membranes isolated from E. coli that coexpress *ompA*(+2)-CYP3A4 and P450 reductase (CPR). **(A)** Typical $Fe^{2+}$ vs $Fe^{2+}$-CO difference spectrum with main absorption at 450 nm. The absorption at 420 nm varies from preparation to preparation and is most likely caused by other sources containing heme. **(B)** Immunoblot analysis of human liver microsomes (lane 1) and membranes isolated from *E. coli* that coexpress *ompA*(+2)-CYP3A4 and P450 reductase (lane 2). First antibodies are directed against CYP3A4 and against P450 reductase. Reductase has a lower mobility than CYP3A4 and is not detected in human liver microsomes, most likely owing to its lower level compared with that found in the bacterial membranes. **(C)** Quantification of P450 content in whole cells or membranes and of P450 reductase in membranes. In addition, testosterone 6β-hydroxylase activity was determined in membranes. The turnover number ($min^{-1}$) is similar to that estimated for CYP3A4 in human liver microsomes.

2. Discard the supernatant and resuspend the cell pellet in ice-cold 2X TSE (5 mL/100 mL of culture); then dilute with an equal volume of ice-cold water. At this point, an aliquot of cells may be withdrawn and kept on ice for determination of a P450 spectrum (*see* **Subheading 3.4.1.**).
3. Add lysozyme to the resuspended cells to a final concentration of 0.25 mg/mL (i.e., dilute the stock solution 80-fold); then shake or stir gently at 4°C for 30–60 min.
4. Pellet the spheroplasts (2800*g*, 20–25 min, 4°C) and discard the supernatant. Resuspend in ice-cold spheroplast resuspension buffer. Use 4 mL of buffer for a pellet derived from 100 mL of culture. At this point, the spheroplasts are usually stored at –70°C (*see* **Note 16**). However, it is possible to continue with isolation of the membrane fraction, as follows.
5. Thaw the spheroplasts on ice (if previously frozen). Add the protease inhibitors aprotinin and leupeptin to a final concentration of 1 μg/mL, and PMSF to a final concentration of 1 m*M*.
6. Sonicate in 30-s bursts while trying to keep the suspension as cold as possible. A total of about 90 s is usually sufficient for spheroplasts deriving from 100–125 mL of culture (*see* **Note 17**).
7. Divide the suspension into 1.5-mL microcentrifuge tubes and spin at top speed (about 12,000*g*) for 12 min at 4°C.
8. Pipet the supernatant into ultracentrifuge tubes, topping up with buffer if necessary (*see* **Note 18**). Spin for 60 min at 180,000*g* (4°C) (*see* **Note 19**).
9. Discard the supernatant and resuspend the membranes in ice-cold 1X TSE buffer (*see* **Note 20**). Use 1 mL of buffer for a pellet derived from 100 mL of culture. Then pour the pellet into a 1-mL Dounce tissue grinder (Wheaton) and disperse using 8–10 strokes of the pestle. Store the membranes at –70°C (*see* **Note 21**).

## 3.4. Analytical Procedures for Quantification of P450 and P450 Reductase

### 3.4.1. Spectral Determination of P450

1. Add 125 μL of sample (whole cells or membranes) to 2.38 mL of 1X P450 spectrum buffer (*see* **Notes 22** and **23**) in a bijou (or similar) and swirl gently. Add a few grains of sodium dithionite (*see* **Note 24**) and mix gently by inversion. Divide equally between a pair of matched 1-mL stoppered optical-glass cuvets.
2. Run a baseline scan from 500 to 400 nm (*see* **Notes 25** and **26**).
3. In a fume hood, bubble the sample cuvet gently (about 1 bubble/s) with CO for 45–60 s.
4. Repeat the scan from 500 to 400 nm. Remove both cuvets from the spectrophotometer, mix gently by inversion, and run another scan (*see* **Notes 27** and **28**). Measure the size of the peak at 450 nm (use the absorbance at 490 nm as reference), and quantify using the following extinction coefficient: $e_{cytP450} = 0.091\ \mu M^{-1}\ cm^{-1}$.

### 3.4.2. Cytochrome-c Reductase Assay

1. Set the spectrophotometer to time scan at a wavelength of 550 nm. Switch on the cuvet heater to 37°C.
2. To two matched glass cuvets, add 0.99 mL of cytochrome-*c* assay buffer.
3. To both cuvets, add 1–10 μg of the protein sample to be tested (i.e., 1–10 μL of a 1 mg/mL solution). Mix (*see* **Note 29**).
4. Ensure that the absorbance remains at zero for approx 1 min.
5. Add 10 μL of 5 m*M* NADPH only to the sample cuvet. Mix.
6. Record the change in absorbance over at least 3 min. The activity should be linear. Calculation of the activity is based on the extinction coefficient for reduced cytochrome-*c*, which is 21.4 $mM^{-1}\ cm^{-1}$ at 550 nm.

### *3.5. Results Achieved for the Expression of P450s*

The results achieved for the bacterial expression of P450s in *E. coli* are exemplified in **Fig. 3** using as an example the isoform CYP3A4. As seen in **Fig. 3B**, the amount of bacterially expressed CYP3A4 is distinctly higher than the level found in human liver microsomes. In this subcellular fraction the amount of CYP3A4 is typically 10–100 pmol/mg of protein, whereas the level of CYP3A4 in bacterial membranes was found to be 270 pmol/mg protein (**Fig. 3C**). The turnover number ($min^{-1}$) of the bacterially expressed CYP3A4 was similar to the value calculated for human liver microsomes.

## 4. Notes

1. Other strains of *E. coli* that have been used successfully by others for expression of P450 include XL-1 Blue and TOPP3 (Stratagene).
2. It is important to ensure that the bacterial *ompA* and *pelB* leader sequences are removed by bacterial signal peptidase, in order to optimize the coupling between the P450 and P450 reductase. We have demonstrated that retained signal peptides can lead to a reduction in catalytic activity. The efficiency of signal peptide removal depends on the amino acid sequence immediately surrounding the cleavage site, which includes the first few amino acids at the P450 N-terminus. Although the sequence of some mammalian P450s allows efficient removal of an *ompA* leader fused directly to the P450 N-terminus during bacterial expression, this is not the case for all P450s. The introduction of the first two amino acids of the mature OmpA protein (Ala-Pro) between the signal peptide and the P450 (the *ompA*+2 strategy) allows for efficient cleavage of the leader sequence, whatever the P450 N-terminal sequence. A prediction for signal cleavage of any *ompA* leader–P450 fusion protein can be obtained at www.cbs.dtu.dk/services/SignalP/ (the SignalP 3.0 server).
3. The restriction enzyme *Bcl*I will not cut plasmid DNA isolated from most common laboratory strains of *E. coli* because its action is blocked by Dam methylation of DNA. The plasmid must therefore be passed through a *Dam*$^-$ strain (such as SCS110, available from Stratagene) first.
4. For the best yields of P450, use only colonies picked from fresh plates to inoculate the starter culture. Frozen cell stocks can be used, although the level of expression may be lower.
5. Do not leave the starter culture at 37°C for more than 16 h, because stationary-phase cells can give lower expression.
6. It is important to autoclave the phosphate solution separately when making up TB from its individual components. Try not to autoclave the broth for longer than necessary, because this can lead to caramelization (the broth becomes very dark), which can be associated with lower P450 yields. If in a hurry, it is possible to sterilize the TB by filtration, but autoclaving is generally recommended. Several manufacturers (Merck, Fluka) offer complete TB ready mixed in a powdered or granular format; in our hands this gives an acceptable level of P450 expression.
7. The inclusion of trace elements in the TB is not essential for P450 expression. This is probably also true for thiamine, although some researchers recommend including it for expression experiments using JM109.
8. The ratio of medium:flask volume can influence the final yield of P450. We routinely use a 1-L conical flask for a culture volume of 100–125 mL.
9. If preferred, the expression culture can be incubated initially at 37°C. This will reduce the time to induction. In this case, it is important that the temperature of the culture be returned to 30°C *before* the addition of IPTG.

10. The optimal phase of bacterial cell growth for induction of P450 expression appears to be mid- to late logarithmic. Inducing too early or too late can markedly affect the yield of P450.
11. IPTG is an expensive chemical. Reducing the final concentration of IPTG to 0.5 m*M* seems to have little effect on P450 yield.
12. Addition of the heme precursor ALA usually improves the yield of spectrally active P450, presumably owing to its effect on heme biosynthesis. The concentration of ALA required for maximal expression varies among individual P450s and thus should be optimized. *E. coli* strains such as JM109 and DH5α are unable to take up heme directly from the culture medium.
13. Incubation temperature is the most important single factor for successful expression of mammalian P450s in *E. coli*. The optimal expression temperature tends to be between 28 and 32°C.
14. Shaking speed may also require optimization; a range of 120–200 rpm is suggested.
15. The induction time should be optimized for each P450. Expression can be monitored by running P450 spectra on samples withdrawn from the incubation at different times. Typical length of induction is 1 to 2 d.
16. The procedure described herein for the isolation of spheroplasts is somewhat harsh and can cause cells to lyse during resuspension (as evidenced by an increase in viscosity). This can make the preparation extremely difficult to manipulate with a pipet, but does not appear to affect the final yield of P450. If desired, PMSF can be added to the resuspended spheroplasts at this stage, before freezing, rather than during thawing before preparation of membranes.
17. It is not necessary to wait until the spheroplasts are completely thawed before starting the sonication. We use an MSE Soniprep 150 machine on 70% power (although this has not been optimized). During sonication the viscosity of the solution will initially increase, as cells lyse and DNA is released, but one should then find that the viscosity decreases again. Keep the tip of the sonicator horn well below the surface of the liquid to avoid creating too much frothing.
18. About 30–35% of the total spectrally active P450 is usually lost in the 12,000*g* pellet. For the highest yields of P450, it is possible to resuspend the 12,000*g* pellet in fresh buffer and repeat the sonication step, but the small amount of extra P450 recovered is not usually worth the effort.
19. We use 6-mL crimp-top polyallomer ultracentrifuge tubes (Sorvall) in a TFT 45.6 rotor spinning at 42,000 rpm in a Sorvall OTD65B ultracentrifuge.
20. We find it easiest to use a heat-sealed glass pipet to dislodge the membrane pellet from the wall of the tube into the buffer.
21. The final protein concentration is typically 25–30 mg/mL, based on a Bradford dye-binding assay, with bovine serum albumin as standard.
22. Dilute the 2X P450 spectrum buffer with an equal volume of water before use. Store at 4°C.
23. We have found that using P450 spectrum buffer for determination of CO spectra in *E. coli* samples gives much more satisfactory results (in terms of the speed of CO-complex formation and reproducibility) than using a buffer such as 0.1 *M* Tris-HCl, pH 7.4. However, we do not recommend the use of this buffer for determination of P450 in liver microsomal samples, because this will lead to an underestimation of the P450 concentration.
24. Less dithionite is needed to reduce the P450 in membrane samples compared with whole cells. Use of too much dithionite can lead to P450 denaturation.

25. The best spectra are obtained by running a “reduced” baseline (i.e., after reduction with dithionite), rather than an “oxidized” baseline.
26. Spectrophotometers from some manufacturers are only able to scan in the opposite direction (i.e., from 400 to 500 nm). This does not appear to affect the estimation of P450 concentration. A scale of –0.02 to +0.02 absorbance units is usually sufficient for whole-cell spectra. For membranes, –0.05 to +0.05 absorbance units or greater may be required.
27. Although reduction of most microsomal P450s with dithionite occurs fairly rapidly in isolated membrane fractions, this is not always the case. For example, the complete reduction of CYP4A11 can take up to 20–30 min. Scans must therefore be repeated until the formation of the reduced-CO complex is complete (maximal size of peak at approx 450 nm).
28. A significant peak of absorbance is often found at 420 nm in whole-cell samples. We have taken this to represent denatured P450 (termed cytochrome P420). However, this peak is likely to overlap the absorbance maximum of endogenous *E. coli* cytochrome-*o* at 416 nm. The peak at 420 nm is usually much reduced in membrane samples.
29. For greater accuracy, the sample can be diluted into the cytochrome-*c* solution before dividing between the two cuvets.

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# 4

## Purification of Cytochromes P450

### *Products of Bacterial Recombinant Expression Systems*

**F. Peter Guengerich and Martha V. Martin**

**Summary**

A general procedure for the solubilization of cytochrome P450 (P450) from bacterial membranes specifically for a human P450 expressed heterologously in the host *Escherichia coli* is described. The example involves the use of a P450 (3A4) with a C-terminal oligohistidine tag and includes sequential DEAE and metal affinity chromatography.

**Key Words:** P450 3A4, chromatography; metal affinity chromatography; DEAE; *Escherichia coli*; heterologous expression; purification; solubilization.

## 1. Introduction

A procedure is outlined that is used for purification of heterologously expressed cytochrome P450 (P450) 3A4 from *Escherichia coli* membranes in our laboratory. Details of the construction of the expression plasmid (NF14) are presented elsewhere ***(1,2)***, including the addition of the C-terminal pentahistidine ($[His]_5$) tag ***(3,4)***. We have described previously the use of traditional methods for the purification of P450s expressed in bacterial systems ***(5)***. Here we describe the incorporation of the $(His)_5$ tag and the use of metal affinity chromatography to purify the recombinant protein.

Attachment of an oligo-His region, usually at the N- or C-terminus, has been used to facilitate protein purification ***(6)***. The free His residues (usually $[His]_4$, $[His]_5$, or $[His]_6$) can chelate $Ni^{2+}$ and similar metal ions; thus, an $Ni^{2+}$-chelate affinity column can be used for rapid purification ***(7)***. Such approaches have been used with P450s, with His tags at either the C- ***(8,9)*** or N- ***(10,11)*** terminus. Detergent is needed to solubilize the membranes and keep the proteins disaggregated during chromatography, and the detergent must be removed in a subsequent step.

In our early work on the purification of *E. coli*-expressed human P450s, the procedures consisted mainly of ion-exchange chromatography methods ***(1,2,5,12–15)***. The change to metal affinity methods was made for two reasons: (1) metal affinity approaches can be

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used to reduce the need for nonionic detergents, or to facilitate removal of these (nonionic detergents can yield artifacts and are even substrates *[16]*) and (2) some P450 mutants are relatively unstable and their purification requires more rapid methods *(17,18)*.

An example is provided in which an ion-exchange step is used before metal affinity chromatography *(3)*. In some cases the step can be omitted and solubilized P450 preparations can be used directly for metal affinity chromatography (e.g., P450s 1A2 and 2D6 *(11,17–20)*.

## 2. Materials

### 2.1. Preparation of Bacterial Membranes

1. Buffer A: 0.10 *M* Tris-acetate buffer (pH 7.6) containing 0.50 *M* sucrose and 0.5 m*M* ethylenediaminetetraacetic acid (EDTA).
2. Lysozyme (50 mg/mL).
3. Buffer B: 0.10 *M* potassium phosphate buffer (pH 7.4) containing 6 m*M* magnesium acetate, 20% (v/v) glycerol, and 10 m*M* 2-mercaptoethanol.
4. 0.1 *M* phenylmethylsulfonyl fluoride (PMSF), in *n*-propanol (stored at –20°C).
5. 0.20 m*M* leupeptin in $H_2O$.
6. 0.1 m*M* bestatin in $H_2O$.
7. Aprotinin (4 U/mL).

### 2.2. Solubilization and Chromatography of P450 3A4 With Histidine Tag

1. 3-([3-Cholamidopropyl]dimethylammonio)-1-propanesulfonic acid (CHAPS), Sol-grade (Anatrace, Maumee, OH).
2. Buffer C: 100 m*M* potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 6 m*M* magnesium acetate, and 10 m*M* 2-mercaptoethanol.
3. 0.10 *M* sodium EDTA (pH 7.5).
4. DEAE-Sepharose, adjusted to pH 7.4 (Amersham, Piscataway, NJ) (*see* **Note 1**).
5. Ni-nitrilotriacetate (NTA) agarose (Qiagen, Valencia, CA) (*see* **Note 2**).
6. Buffer D: 20 m*M* potassium phosphate buffer (pH 7.4) containing 10 m*M* 2-mercaptoethanol, 20% (v/v) glycerol, and 1% (w/v) CHAPS.
7. Buffer E: 20 m*M* potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 0.5% CHAPS (w/v), 0.5 *M* KCl, and 10 m*M* 2-mercaptoethanol.
8. Buffer F: 20 m*M* potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 0.5 *M* KCl, and 10 m*M* 2-mercaptoethanol.
9. Buffer G: 20 m*M* potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 0.5 *M* KCl, 10 m*M* 2-mercaptoethanol, and 400 m*M* imidazole. Recheck the pH and adjust to 7.4 with 43% (or more dilute) $H_3PO_4$.
10. Buffer H: 100 m*M* potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol, 0.1 m*M* EDTA, and 0.1 m*M* α-dithiothreitol.

## 3. Methods

All steps are done at 0–4°C.

### 3.1. Preparation of Bacterial Membranes

1. Recover bacterial cells by centrifuging (4000*g* for 15 min) and resuspend in buffer A at a concentration of approx 70 mg of wet cells/mL.

2. Dilute the suspension twofold with $H_2O$ and add lysozyme to 0.1 mg/mL.
3. Gently shake the suspension and incubate on ice for 30 min in order to hydrolyze the outer membranes.
4. Recover the resulting spheroplasts by centrifuging at 40,000*g* for 15 min; resuspend the pellet in buffer B at a concentration of ~0.5 g/mL. At this point the preparation can be stored frozen at –70°C until further use ***(2)***.
5. Thaw the frozen spheroplasts in a water bath at room temperature. During the thawing process, add protease inhibitors to the spheroplasts to the following final concentrations: 1.0 m*M* PMSF, 2.0 μ*M* leupeptin, 1.0 μ*M* bestatin, 0.04 U/mL of aprotinin.
6. Lyse cells in a Rosette cell packed in ice for approx 15 min (at approx 70% full power) using a Branson sonicator or until the cell lysate contains no clumps.
7. Subject the lysate to centrifugation (10,000*g*, 20 min) and discard the pellet.
8. Cetrifuge the supernatant at 100,000*g* for 90 min (e.g., 35,000 rpm in a Beckman 45 Ti rotor).
9. Resuspend the pelleted membranes in a minimum volume of buffer C, quickly freeze in liquid $N_2$, and store at –70°C (unless further purification is started immediately).

### 3.2. Membrane Solubilization and DEAE Chromatography

1. Dilute the *E. coli* membranes prepared as in **Subheading 3.1.** to a 20 m*M* potassium phosphate concentration by adding of 4 vol of a solution of 20% glycerol (v/v), 1.25% CHAPS (w/v), and 10 m*M* 2-mercaptoethanol. Add buffer D, as necessary, to dilute to a protein concentration of approx 2.0 mg/mL (*see* **Note 3**).
2. Stir the mixture gently for 2–4 h and centrifuge at 100,000*g* for 30 min; discard the pellet.
3. Apply the resulting supernatant to a 2.5 × 10 cm column of DEAE-Sepharose (suitable for approx 1000 mL of solubilized membranes) that has been equilibrated with buffer D, and collect 10 mL fractions.
4. After all of the sample has been applied, wash the column with approx 2 bed vol of buffer D, and pool the fractions containing red (P450). Add KCl (solid) to the pooled fractions to achieve a concentration of 0.5 *M*.
5. Conduct a spectral assay ***(21,22)*** to estimate recovery, and it may be useful also to monitor the progress of purification at this point by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) ***(22,23)*** (*see* **Note 4**).

### 3.3. Nickel Affinity Chromatography

1. Apply the pooled material directly to a 1.5 × 5 cm column of Ni-NTA agarose (equilibrated with buffer E) at a flow rate of approx 1 mL/min. Most of the brown color should be adsorbed to the column.
2. Wash the column with 10 column vol of buffer E.
3. Wash the column with 10 bed vol of buffer F.
4. Elute the protein (P450) from the column with buffer G, and collect 5-mL fractions.
5. Analyze the fractions for $A_{417}$ and by SDS-PAGE, taking care to dilute samples to avoid potassium dodecyl sulfate precipitates.
6. Pool and dialyze extensively (four times, >6 h each time) with buffer H, either before or after concentration with an Amicon ultrafiltration system and a PM-30 membrane (*see* **Note 3**), the P450 fractions (as judged by $A_{417}$) that are homogeneous (>95%) as judged by SDS-PAGE. Estimate the concentration of P450 spectrally ***(21,22)***.

### 3.4 Other Procedures for Purification of P450s From Bacterial Expression Systems

#### 3.4.1. Direct Metal Affinity Chromatography

In some cases, such as with human P450 1A2, we have proceeded directly from preparation of solubilized membranes to metal affinity chromatography *(11,17–20)*. In these cases, highly purified P450s can be prepared without the need for earlier steps.

#### 3.4.2. Ion-Exchange Chromatography Systems

P450 1A2 has been purified from bacterial membranes using a combination of only DEAE chromatography and carboxymethylcellulose (CM) chromatography *(24)*. Sometimes a two-step system is sufficient to purify the protein and remove detergent (e.g., P450 1A2 *[24])*. In other cases, a sequence of DEAE/CM/hydroxylapatite has been used *(1,12–15)*.

#### 3.4.3. Phase Separation

Some detergents have temperature-dependent cloud points. It is possible to fractionate protein mixtures between the "detergent" and "soluble" phases with some detergents, such as Triton X-114 *(25,26)*. This approach is relatively easy and has been successfully applied to bacterial P450 fractionation to achieve partial purification *(8,27,28)*.

#### 3.4.4. Flavodoxin Affinity Chromatography

Jenkins and Waterman *(8)* reported that *E. coli* flavodoxin is the source of electrons used by recombinant P450s expressed in the bacteria. They also found that an affinity column made of immobilized flavodoxin could selectively bind mammalian P450s *(8)*. This approach has been utilized in the purification of recombinant P450 2D6 *(27)*. Bacterial membranes were subjected to the phase separation described in **Subheading 3.4.2.** and then applied to a flavodoxin affinity column, which was eluted with a gradient of increasing NaCl concentration in the presence of nonionic detergent *(8,27)*.

#### 3.4.5. Fusion Proteins

Two types of P450 fusion proteins have been constructed and purified. P450:NADPH-P450 reductase fusion proteins and close relatives occur naturally *(29–31)* and have facilitated internal electron transfer. Similar artificial constructs were first prepared in yeast *(32)* and later in bacteria *(33)*. Purification was facilitated by the ability to use 2',5'-ADP agarose affinity chromatography on the reductase portion. The typical procedure involves initial separation on DEAE, followed by the 2',5'-ADP column, plus steps to remove detergents. This approach has been used to purify proteins containing human P450s 1A1, 1A2, and 3A4 fused with the reductase *(34–36)*, as well as some animal P450s *(33,34,37)*.

Another kind of fusion protein has glutathione-*S*-transferase (GST) attached to the N-terminus of a P450 (2B4) *(38)*. The presence of a GST renders the protein soluble and enables the use of a glutathione-based affinity matrix for purification *(39)*. The construction of a thrombin-sensitive site between the GST and P450 domains facilitates the release of the GST portion to cleave the P450.

## 4. Notes

1. *See* **ref. *5*** regarding substitution of other chromatography media.
2. An alternative is a $Co^{2+}$-based matrix, such as the Talon® system (Clontech/BD Biosciences, Palo Alto, CA), although we have not used this in our own work.
3. Some P450s are not solubilized well with only an ionic detergent (e.g., CHAPS, cholate) and require the addition of a nonionic detergent (e.g., Triton N-101, Emulgen 911 or 913, Tergitol NP-10) ***(5)***. P450s in our category that we have encountered in this laboratory include P450 2A6 and 2D6.
4. *See* **ref. *5*** regarding dialysis and various methods of protein concentration.

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# 5

# Expression of Recombinant Flavin-Containing Monooxygenases in a Baculovirus/Insect Cell System

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**Summary**

The baculovirus/insect cell heterologous expression system provides an important tool for investigating the catalytic activity of individual drug-metabolizing enzymes toward a particular substrate. In this chapter we describe a baculovirus/insect cell system that we have used for the expression of human and mouse flavin-containing monooxygenases. Methods are described for the generation of recombinant baculoviral DNAs, via both site-specific transposition in *Escherichia coli* and site-specific recombination in vitro; adaptation of *Spodoptera frugiperda* (*Sf*) 9 cells to shaking culture and to serum-free medium; cryopreservation and transfection of *Sf*9 cells; amplification of baculovirus and determination of viral titer; analysis of baculoviral DNA; and expression and analysis of recombinant proteins.

**Key Words:** Flavin-containing monooxygenase, FMO4; cytochrome P450; heterologous expression; baculovirus; insect cells.

## 1. Introduction

The expression of a cDNA clone in a heterologous system, such as bacterial, yeast, insect, or mammalian cells, is a convenient means of obtaining relatively large amounts of the encoded protein for structural or functional studies. The baculovirus/insect cell system allows high levels of expression and is capable of correct posttranslational modification and subcellular targeting of the expressed protein. The system is particularly suitable for the expression of phase I drug-metabolizing enzymes, such as cytochromes P450 (CYPs) and flavin-containing monooxygenases (FMOs), because it ensures that the proteins are expressed in an appropriate environment, the endoplasmic reticulum (ER) of a higher eukaryote. The host insect cells have very low amounts of endogenous CYPs and FMOs and, thus, mammalian CYPs and FMOs expressed in the baculovirus system can often be studied in isolated microsomes, without the need for further purification. In the case of FMOs, the activities of heterologously expressed proteins can be measured indirectly by

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monitoring consumption of the cofactor nicotinamide adenine dinucleotide phosphate (NADPH), because the insect cells have very low NADPH-oxidase activity.

Many members of the CYP and FMO families have broad, and sometimes overlapping, substrate specificities. Determination of the kinetic parameters, e.g., $K_M$, $V_{max}$, and $k_{cat}$, of reactions catalyzed by heterologously expressed CYPs and FMOs can help identify the isoform(s) that contribute to the metabolism of a particular drug or other foreign or endogenous chemical. For instance, clozapine, an atypical antipsychotic, is *N*-oxygenated by CYP3A4 and FMO3 ***(1)***, and tazarotenic acid, a metabolite of tazarotene, undergoes *S*-oxygenation by FMO1, FMO3, and CYP2C8 ***(2)***. By contrast, FMO1 and FMO3, but not CYPs, catalyze the *N*-oxygenation of itopride, a gastroprokinetic agent ***(3)***, and of benzydamine ***(4)***, whereas the *N*-oxygenation of trimethylamine is catalyzed by FMO3 ***(4a)***. It has also been possible, using this approach, to distinguish the catalytic specificities of three closely related members of the CYP2C subfamily of humans, CYP2C8, CYP2C9, and CYP2C19. CYP2C8 catalyzes paclitaxel 6α-hydroxylation ***(5,6)***; CYP2C9, diclofenac 4'-hydroxylation ***(6)***; and CYP2C19, (*S*)-mephenytoin 4'-hydroxylation ***(6)***. Although analysis of heterologously expressed proteins will indicate the ability of an individual enzyme to catalyze a particular reaction on a given compound, it will not definitively establish the role of the enzyme in the metabolism of the compound in vivo. For this it is necessary to take into account the amount and tissue distribution of the enzyme, and to carry out experiments on isolated human microsomes, preferably using isoform-specific chemical or immunoinhibitors (*see*, e.g., **ref. *6***) (*see* Chapter 7 for discussion), or on mouse lines deficient in particular drug-metabolizing enzymes (*see*, e.g., Chapter 34).

The baculovirus/insect cell system has also been used to determine the functional consequences of mutations and genetic polymorphisms of CYPs and FMOs. For instance, analysis of the catalytic activities of heterologously expressed FMOs has identified loss-of-function mutations of FMO3 that are causative of the inherited disorder trimethylaminuria (or fish-odor syndrome) ***(7–9)***; established that the major FMO2 allele of humans encodes a truncated, inactive protein ***(10)***; and determined the effect on function of genetic polymorphisms of human FMOs ***(7,8,11)***.

Baculoviruses are composed of a circular, double-stranded DNA genome contained in a rod-shaped capsid. They infect insects, particularly those of the order Lepidoptera. The most common baculovirus used for heterologous expression is the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV). The virus is propagated in host cells derived from the fall armyworm *Spodoptera frugiperda* (*Sf*) or the cabbage looper *Trichoplusia ni*.

Recombinant baculoviruses were originally generated by homologous recombination in host cells. This was achieved by cotransfecting insect cells with wild-type (WT) AcMNPV DNA and a transfer vector containing the cDNA of interest, under the control of a strong baculovirus promoter, flanked by baculoviral DNA derived from a nonessential locus. This approach has several disadvantages: a very low proportion of the viruses generated is recombinant, recombinant and WT viruses are distinguished on the basis of subtle differences in plaque morphology, and several rounds of plaque purification are necessary to obtain the desired recombinant.

Quicker and more efficient methods of generating recombinant baculoviruses have subsequently been developed. In the Bac-to-Bac system (available from Invitrogen, Paisley, UK), recombinant viral DNA is produced via Tn7-mediated site-specific transposition in *Escherichia coli* (*12*). Transposition disrupts the *lacZ*α gene and, thus, bacterial colonies containing recombinant baculoviral DNA can be selected by the blue-white assay. Recombinant DNA can then be isolated and used to transfect insect cells to generate recombinant baculovirus. In the BaculoDirect system (available from Invitrogen), recombinant baculoviral DNA is produced in vitro by recombination between specific *att* sites. Recombination results in the cDNA of interest replacing a thymidine kinase (TK) gene present in the BaculoDirect DNA. After subsequent transfection, insect cells are cultured in the presence of ganciclovir, which enables negative selection against cells that contain nonrecombinant baculovirus.

## 2. Materials

### 2.1. Generation of Recombinant Bacmid DNA by Site-Specific Transposition in E. coli

#### 2.1.1 Construction of Donor Plasmid

1. pFastBac plasmid (Invitrogen): this plasmid comes in two forms, either with an N-terminal 6X Histidine affinity tag (6X His tag) (available in all three reading frames) or without a tag. Vectors can be purchased as part of the Bac-to-Bac baculovirus expression system kit.
2. FMO cDNA.
3. Competent *E. coli* strain DH5α (available in aliquots of 50 μL, ready for transformation).
4. Oligonucleotide primers.
5. Proofreading, thermostable DNA polymerase, e.g., BIO-X-ACT *Taq* polymerase (Bioline, London, UK).
6. QIAquick Gel Extraction Kit (Qiagen, Crawley, UK).
7. Restriction endonucleases and T4 DNA ligase (New England Biolabs, Hitchin, UK).
8. QIAprep Spin Miniprep Kit (or any similar small-scale plasmid isolation kit).
9. Agarose (Invitrogen).
10. Hyperladder™ I (Bioline).
11. Luria-Bertani (LB) medium, SOB medium, and LB-agar (Anachem, Luton, UK): prepare by dissolving the appropriate number of capsules in the desired volume of distilled water and autoclaving.
12. SOC medium: prepare by adding glucose (final concentration of 10 m*M*) to the appropriate amount of autoclaved SOB medium (*see* **item 11**).
13. Ampicillin (Sigma-Aldrich, Poole, UK).

#### 2.1.2. Transposition

1. Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich): 1 *M* in water, filter-sterilized.
2. Bluo-gal™ (Invitrogen): 2% (w/v) in dimethyl sulfoxide (DMSO) (Sigma-Aldrich).
3. LB-agar plates containing kanamycin (50 μg/mL), gentamicin (7 μg/mL), and tetracycline (10 μg/mL) (antibiotics purchased from Sigma-Aldrich).
4. Competent *E. coli* DH10Bac cells (Invitrogen): these cells are available in ready-to-use aliquots of 50 μL.
5. SOC medium (*see* **Subheading 2.1.1., item 12**).

### 2.1.3. Isolation of Recombinant Bacmid DNA

1. LB medium containing kanamycin (50 µg/mL), gentamicin (7 µg/mL), and tetracycline (10 µg/mL).
2. RNase A (10 µg/mL).
3. Alkaline lysis solution I: 50 m*M* glucose, 25 m*M* Tris-HCl (pH 8.0), 10 m*M* ethylenediaminetetraacetic acid (EDTA) (pH 8.0). Autoclave and add RNase A to a final concentration of 100 µg/mL. Store at 4°C.
4. Alkaline lysis solution II: 0.2 *M* NaOH, 1% (w/v) sodium dodecyl sulfate (SDS). Prepare this solution fresh and use at room temperature.
5. Alkaline lysis solution III: 60 mL of 5 *M* potassium acetate, 11.5 mL of glacial acetic acid; make up to 100 mL with deionized water. The resulting solution is 3 *M* with respect to potassium and 5 *M* with respect to acetate. Store at 4°C.
6. Isopropanol.
7. 70% (v/v) Ethanol.
8. 0.8% (w/v) Agarose gel.

## 2.2. Generation of Recombinant Baculoviral DNA by Site-Specific Recombination In Vitro

### 2.2.1. Construction of Entry Vector Clone

1. Linearized pENTR™/D-TOPO® vector, TOPO adapted (Invitrogen): this can be purchased as part of a pENTR/D-TOPO cloning kit, at a concentration of 15–20 ng/µL in 50% (v/v) glycerol, 50 m*M* Tris-HCl (pH 7.4), 1 m*M* EDTA, 2 m*M* dithiothreitol, 0.1% (v/v) Triton X-100; bovine serum albumin (100 µg/mL), 30 µ*M* bromophenol blue.
2. FMO cDNA.
3. Oligonucleotide primers (*see* **Subheading 3.2.1.** for details of design).
4. Thermostable, proofreading DNA polymerase, such as AccuPrime™ *Pfx* (Invitrogen) or BIO-X-Act (Bioline).
5. Thermocycler.
6. Agarose.
7. Gel electrophoresis equipment.
8. Hyperladder I (Bioline).
9. Salt solution: 1.2 *M* NaCl, 0.06 *M* $MgCl_2$ (supplied as part of the TOPO cloning kit).
10. Competent *E. coli* (e.g., TOP10, JM109, or DH5α) in 50-µL aliquots.
11. LB-agar plates containing kanamycin (50 µg/mL).
12. LB medium containing kanamycin (50 µg/mL).
13. QIAprep Spin Miniprep Kit (Qiagen) or any similar small-scale plasmid isolation kit.
14. Restriction endonucleases.

### 2.2.2. Site-Specific Recombination In Vitro

**Items 1–4** are supplied in BaculoDirect Expression kits that can be purchased from Invitrogen.

1. BaculoDirect™ Linear DNA: BaculoDirect C-term, BaculoDirect Secreted, or BaculoDirect N-term (Invitrogen). This can be purchased as part of a BaculoDirect Expression kit (30 ng/vial in 10 µL of TE buffer, pH 8.0).
2. LR Clonase™ Enzyme Mix, a mixture of λ- and *E. coli*-encoded enzymes.

3. 5X LR Clonase reaction buffer.
4. Proteinase K solution: 2 μg/mL in 10 m*M* HCl, pH 7.5, 20 m*M* $CaCl_2$, 50% (v/v) glycerol.
5. TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.

### *2.3. Propagation of* Sf*9 Insect Cells*

1. *Sf*9 cells (Invitrogen).
2. Gentamicin (50 mg/mL solution) (Invitrogen).
3. Fetal calf serum (FCS) (Invitrogen).
4. Serum-containing medium: Sf-900 II serum-free medium (SFM) (Invitrogen) supplemented with 10% (v/v) FCS and gentamicin (50 μg/mL).
5. SFM: Sf-900 II SFM containing gentamicin (10 μg/mL).
6. Dulbecco's phosphate-buffered saline (PBS) without magnesium (Invitrogen).
7. 0.4% (w/v) Trypan blue stain (Invitrogen).
8. Hemocytometer (VWR, Lutterworth, UK).
9. Nonhumidified 27°C incubator (with shaking facility, if shaker cultures are to be used).
10. DMSO.
11. Sterile, disposable Corning Erlenmeyer flasks (125-mL and 250-mL) (if using shaker cultures) and Nunc™ T175 flasks (for monolayer cultures) (VWR).

### *2.4. Cryopreservation of* Sf*9 Cells*

1. *Sf*9 cells in mid-log phase (cell density of $2 \times 10^6$ cells/mL).
2. Cryopreservation medium for serum-free culture: 7.5% (v/v) DMSO in 50% (v/v) fresh SFM and 50% (v/v) conditioned medium (this is medium removed from a 2- to 3-d-old culture and filter-sterilized).
3. Cryopreservation medium for serum-containing cultures: fresh medium supplemented with 7.5% (v/v) DMSO and 10% (v/v) FCS. Chill to 4°C before use.
4. Cryovials (1.5- or 2.5-mL) (VWR).

### *2.5. Transfection of* Sf*9 Cells With Recombinant Bacmid DNA*

1. Mid-log phase culture of *Sf*9 cells (cell density of $2 \times 10^6$ cells/mL).
2. Tissue culture dishes (35-mm) (VWR).
3. SFM with gentamicin (10 μg/mL).
4. Recombinant bacmid DNA.
5. SFM without gentamicin.
6. Solution A: for each transfection, 5 μL of miniprep bacmid DNA (*see* **Subheading 3.1.3.**) or 10 μL of LR recombination reaction mix (*see* **Subheading 3.2.2.**), plus 100 μL of SFM without gentamicin.
7. Solution B: for each transfection, 6 μL of CELLFECTIN™ reagent (Invitrogen) plus 100 μL of SFM without gentamicin. The CELLFECTIN reagent must be mixed thoroughly before use, because the lipid settles at the bottom of the tube.

### *2.6. Viral Amplification*

1. Nunc T175 flasks (VWR).
2. *Sf*9 cells in mid-log phase.
3. SFM containing gentamicin (10 μg/mL).
4. Stock recombinant virus to be amplified.

### 2.7. Viral Plaque Assay

1. Nunc six-well tissue culture dishes (VWR).
2. Mid-log phase culture of *Sf*9 cells.
3. SFM containing gentamicin (10 µg/mL).
4. 4% (w/v) Agarose (Invitrogen).
5. Neutral red (Sigma-Aldrich): dilute neutral red solution in SFM to give a final concentration of 1 mg/mL. Add to SFM containing gentamicin (*see* **item 3**) to give a final concentration of 56 µg/mL.

### 2.8. Analysis of Viral DNA

1. PBS.
2. Recombinant viral DNA.
3. BIO-X-ACT *Taq* DNA polymerase and associated buffer (Bioline).
4. Lysis buffer: 10 m*M* Tris-HCl, pH 8.3, gelatin (100 µg/mL), 0.45% (v/v) Triton X-100, 0.45% (v/v) Tween-20, 50 m*M* KCl.
5. Proteinase K (6 and 10 mg/mL) (Roche, Lewes, UK).
6. TlowE buffer: 10 m*M* Tris-HCl, pH 8.0, 0.1 m*M* EDTA.
7. Cell lysis buffer: 50 m*M* Tris-HCl, pH 8.0, 5% (v/v) 2-mercaptoethanol, 0.4% (w/v) SDS, 10 m*M* EDTA.
8. RNase A (10 mg/mL) (Roche).
9. Phenol/chloroform (1:1 [v/v]).
10. 3 *M* sodium acetate, pH 5.2.
11. Ethanol.
12. 70% (v/v) Ethanol.

### 2.9. Expression and Analysis of Recombinant Protein

1. Culture of *Sf*9 cells 72 h after infection with the relevant recombinant virus.
2. Sonicator.
3. Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich): prepare a 0.2 *M* stock solution in ethanol and store at –20°C.
4. Sonication and resuspension buffer: 10 m*M* sodium phosphate (pH 7.25), 1 m*M* EDTA, 20% (v/v) glycerol, containing 0.4 m*M* PMSF (added just before use).
5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) equipment.
6. Western-blotting apparatus.
7. Anti-His antibody (Qiagen) or an antibody against the expressed protein.
8. Protein standard markers (prestained preferable) (Bio-Rad, Hemel Hempstead, UK).

## 3. Methods

We describe here a baculovirus/insect cell system that we have used for the expression of human and mouse FMOs. Strategies and methods are described for the generation of recombinant baculoviral DNAs, through both site-specific transposition in *E. coli* and site-specific recombination in vitro; adaptation of *Sf*9 cells to shaking culture and to SFM; cryopreservation and transfection of *Sf*9 cells; amplification of baculovirus and determination of viral titer; analysis of baculoviral DNA; and expression and analysis of recombinant proteins. For an excellent overview of general methods for the maintenance of *Sf*9 cells and baculovirus-mediated expression, *see* **ref. *13***.

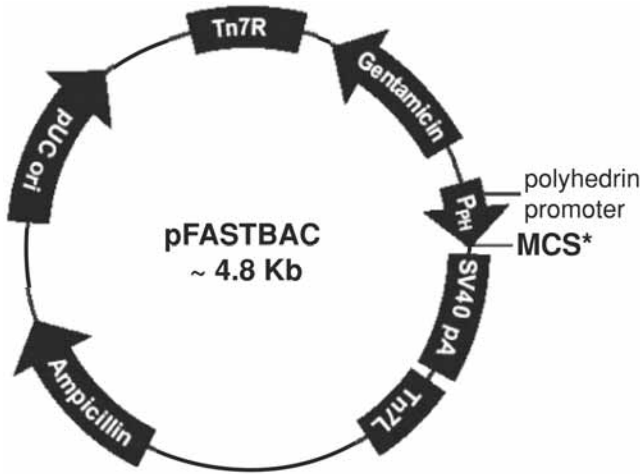


Fig. 1. Diagrammatic representation of main features of donor vector pFastBac 1. MCS, multiple cloning site. In the pFastBac HT series of plasmids, the MCS contains, upstream of various restriction endonuclease sites, an ATG translational initiation codon followed by six histidine codons and a TEV protease-coding sequence.

### 3.1. Generation of Recombinant Bacmid DNA by Site-Specific Transposition in E. coli

#### 3.1.1. Construction of Donor Plasmid

Construction of the donor plasmid involves inserting the relevant cDNA into a pFastBac plasmid (**Fig. 1**). pFastBac plasmids contain a multiple cloning site (MCS), for insertion of the cDNA to be expressed; an ampicillin-resistance gene, for selection of *E. coli* cells harboring the plasmid; an insect cell-specific promoter (e.g., the polyhedrin or p10 promoter from AcNPV), to ensure high-level expression of the cDNA in insect cells; attachment sites for the bacterial Tn7 transposon, which permit site-specific transposition of the cDNA insert from the donor plasmid into a baculovirus shuttle vector (bacmid); a gentamicin-resistance gene, which allows selection of *E. coli* DH10Bac cells containing the recombinant bacmid; and the SV40 poly(A) signal sequence, which ensures efficient transcription termination and subsequent polyadenylation of the expressed mRNA in insect cells.

A convenient method for inserting a cDNA into a pFastBac plasmid is to amplify the cDNA with sequence-specific oligonucleotide primers that also contain appropriate restriction enzyme sites. The polymerase chain reaction (PCR) product is then digested and inserted into the MCS of the plasmid. For example, when constructing the donor plasmid for the human FMO4 cDNA, we designed primers containing *Nco*I sites and cloned the PCR product into the *Nco*I site of the MCS. Alternatively, if appropriate restriction enzyme sites are present in the cDNA, as was the case for mouse FMO4 cDNA, then these may be used.

The basic donor plasmid available in the Bac-to-Bac system (pFastBac 1) uses the ATG initiation codon of the inserted cDNA as a translational start site. The frame in which the cDNA is inserted into this plasmid is thus not relevant. However, if using a donor plasmid containing an N-terminal polyhistidine tag, e.g., pFastBac HT, it is essential that the cDNA be inserted in the correct reading frame. The availability of plasmids in all three reading frames makes cloning in frame easier. The pFastBac HT series of vectors additionally contain a TEV protease cleavage site for removal of the 6X His tag after protein purification. Insertion of the cDNA is carried out using standard molecular biology techniques.

1. If no suitable restriction sites are present within the cDNA, amplify the cDNA with appropriate sequence-specific primers. Electrophorese a small amount of the PCR products through an agarose gel. A single intense band of the correct size should be observed.
2. Purify the remaining PCR product using a QIAquick column per the manufacturer's instructions.
3. Digest the purified PCR product and the relevant pFastBac plasmid (approx 10 μg) with the appropriate restriction enzymes.
4. Purify the digested PCR product, using a QIAquick column, and proceed to **step 8**.
5. If suitable restriction sites are present within the cDNA, digest the cDNA and the pFastBac vector with the relevant enzymes.
6. Electrophorese an aliquot of the digest of the cDNA through an agarose gel to ensure that the insert has been released from the parent vector.
7. Visualize the insert using an ultraviolet illuminator. Using a clean scalpel, cut out the insert from the agarose gel and purify using a QIAquick column as recommended by the manufacturer.
8. Electrophorese a small amount of the purified digested products (*see* **item 4** or **7**) and an aliquot of the digested pFastBac plasmid through an agarose gel. Estimate the amounts of vector and insert using a mass ladder, e.g., Hyperladder I, by comparing the intensity of the fragment in the gel under UV light with that of a standard fragment of the same size in the Hyperladder.
9. Ligate the vector and insert using T4 DNA ligase as recommended by the supplier. Use about a 10:1 molar ratio of insert to vector in a ligation reaction.
10. Transform competent *E. coli* DH5α cells (50 μL) with 2 μL of the ligation mixture, and plate on LB-agar plates containing ampicillin (10 μg/mL). Incubate the plates overnight at 37°C.
11. Inoculate LB medium containing ampicillin (10 μg/mL) with single colonies and grow overnight at 37°C with shaking.
12. Isolate plasmid DNA using a standard method and digest with the appropriate restriction endonucleases. Analyze the digests on an agarose gel to check for the presence of the cDNA. cDNA inserts must be sequenced to ensure that no errors have been introduced during the PCR or cloning procedures.

### 3.1.2. Transposition

Site-specific transposition of the expression cassette into the baculovirus shuttle vector (bacmid) bMON14272 occurs in *E. coli* DH10Bac cells. Recombinant bacmids are constructed by transposing the mini-Tn7 element from the pFastBac donor plasmid to the mini-*att*Tn7 attachment site in the bacmid, with the Tn7 transposition functions provided by a helper plasmid (pMON7124). Insertions of the mini-Tn7 into the

mini-*att*Tn7 attachment site in the bacmid disrupts the *lac*Zα gene, so colonies containing recombinant bacmid are white, whereas nonrecombinant colonies are blue. Recombinant bacmid DNA can be isolated from small-scale cultures and used for transfection of *Sf*9 cells.

1. Transform competent DH10Bac cells (50-μL aliquot) with approx 100 ng of the donor plasmid by heat shock at 42°C for 45 s.
2. Add 900 μL of SOC medium and incubate the cells in a shaking water bath for 4 h.
3. Spread 50 μL of Bluo-gal (0.02 μg) and 10 μL of IPTG (10 μmol) onto each LB-agar plate containing kanamycin (50 μg/mL), gentamicin (7 μg/mL), and tetracycline (10 μg/mL).
4. Prepare serial dilutions ($10^{-1}$, $10^{-2}$, $10^{-3}$) of the transformed cells in SOC medium, and plate 100 μL of each dilution evenly over the surface of the plates described in **step 3**.
5. Incubate the plates for at least 24 h at 37°C, and then for a further 12 h at 4°C, to enhance the color of the blue colonies.

### *3.1.3. Isolation of Recombinant Bacmid DNA*

White colonies, obtained as described in **Subheading 3.1.2.**, contain the recombinant bacmid. Bacmid DNA is isolated using the method of alkaline lysis ***(14)***.

1. Inoculate a single bacterial colony into 5 mL of LB medium containing kanamycin (50 μg/mL), gentamicin (7 μg/mL), and tetracycline (10 μg/mL). Incubate overnight at 37°C with shaking.
2. Transfer 1.5 mL of the culture into a 1.5-mL microfuge tube and centrifuge at 14,000*g* for 10 min at 4°C.
3. Remove the supernatant and resuspend in 300 μL of alkaline lysis solution I (*see* **Subheading 2.1.3., item 3**) by pipetting up and down.
4. Add 300 μL of alkaline lysis solution II (*see* **Subheading 2.1.3., item 4**) and mix gently. Incubate at room temperature for 5 min.
5. Slowly add 300 μL of alkaline lysis solution III (*see* **Subheading 2.1.3., item 5**) and mix gently.
6. Centrifuge for 10 min at 14,000*g*. Transfer the supernatant to a fresh 2-mL microfuge tube and add 800 μL of isopropanol. Mix by gently inverting the tube a few times and place on ice for 5–10 min.
7. Centrifuge at 14,000*g* for 15 min at room temperature.
8. Remove the supernatant, add 500 μL of 70% ethanol, and invert the tube several times to wash the pellet.
9. Centrifuge for 15 min at 14,000*g* at room temperature. Remove the supernatant and air-dry the pellet.
10. Dissolve the DNA in sterile deionized water. Do not vortex to dissolve the DNA, because this will shear it.
11. To avoid repeated freezing and thawing, store the DNA in aliquots at –20°C.

Electrophorese bacmid DNA through a 0.8% agarose gel to confirm isolation (*see* **Note 1**).

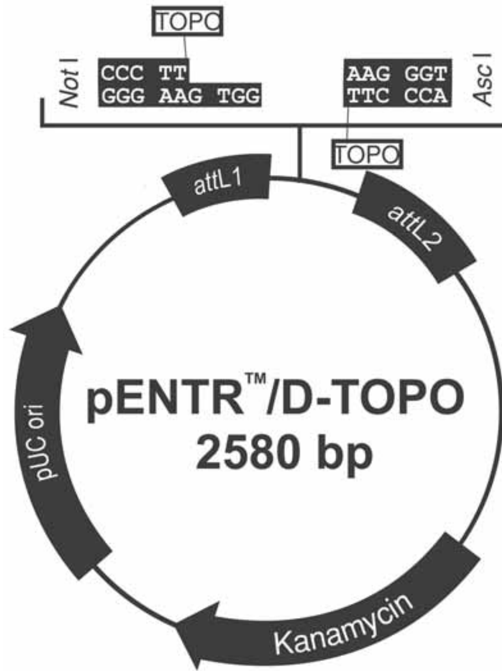


Fig. 2. Entry vector pENTR/D-TOPO.

### *3.2. Generation of Recombinant Baculoviral DNA by Site-Specific Recombination In Vitro*

Another way of generating recombinant baculoviral DNA is via site-specific recombination in vitro. This is the basis of the BaculoDirect system, available from Invitrogen. In this system, the cDNA to be expressed is first inserted into the cloning site of an "entry" vector, such as pENTR/D-TOPO (**Fig. 2**), where it is flanked by *att*L sites, derived from bacteriophage λ. The cDNA insert is then transferred from the entry vector into BaculoDirect linear DNA via site-specific recombination in vitro between the *att*L sites in the entry vector and *att*R sites in the baculoviral DNA (**Fig. 3**). The *att*R sites flank a herpes simplex virus TK gene. Recombination results in replacement of the TK gene with the cDNA insert from the entry vector, thus generating recombinant baculoviral DNA that lacks the TK gene. After transfection with the products of the recombination reaction, insect cells that contain nonrecombinant baculovirus can thus be selected against by culturing in medium containing ganciclovir.

#### *3.2.1. Construction of Entry Vector Clone*

Several entry vectors are available from Invitrogen. The basic vector, pENTR/D-TOPO (**Fig. 2**), contains two recognition sites (CCCTT) for topoisomerase I from *Vaccinia* virus (TOPO recognition sites) flanked by two *att*L sites. The vector is supplied as a linear topoisomerase (TOPO)-charged DNA that facilitates directional cloning of

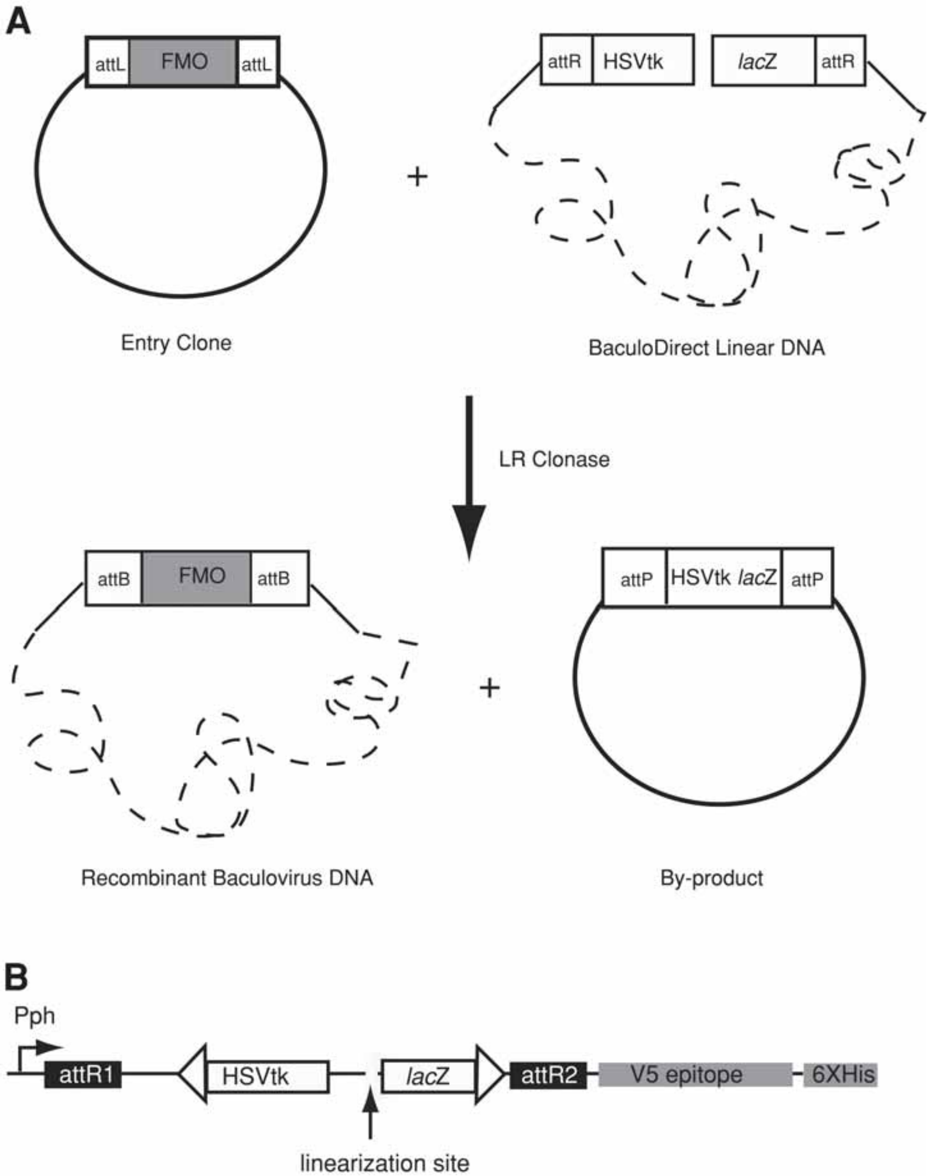


Fig. 3. **(A)** Site-specific recombination between *att*L sites in entry clone and *att*R sites in BaculoDirect linear DNA. **(B)** Map of Gateway™ cassette elements of BaculoDirect C-term linear DNA. Pph, polyhedrin promoter; *att*R1 and *att*R2, recombination sites; HSVtk, herpes simplex virus thymidine kinase gene.

blunt-ended PCR products (TOPO cloning). Primers for PCR amplification of the cDNA of interest must be designed to facilitate both directional cloning and subsequent expression. The forward PCR primer *must* contain, at its 5' end, the sequence CACC, which will basepair with the overhang sequence GTGG in the pENTR/D-TOPO vector (**Fig. 2**), thus ensuring directional cloning of the PCR product. If the PCR product is to be expressed as a native or C-terminal fusion-tagged protein, then the four bases CACC should be followed directly by sequence-specific bases starting from the ATG translational initiation codon. The ATG initiation codon will thus be within the context of a Kozak sequence (an A at position –3), which will favor correct translational initiation.

To ensure efficient directional cloning, the 5' end of the reverse PCR primer must not be complementary to the overhang sequence GTGG; there should be at least two mismatches. If the PCR product is to be expressed without a C-terminal tag, the reverse primer should include the stop codon of the native sequence or bind downstream of the stop codon. If the PCR product is to be expressed with a C-terminal tag, then the reverse primer should be designed to remove the native stop codon; that is, its 5' end should be complementary to the last amino acid codon.

1. Amplify the sequence to be expressed using appropriately designed forward and reverse primers (as previously discussed) and a proofreading DNA polymerase. To ensure that PCR products are completely extended, use a final extension period of 7–30 min.
2. Analyze a small amount of the PCR products by agarose gel electrophoresis. A single, discrete band of the correct size should be observed. Estimate the concentration of the PCR product by comparing with a mass ladder, e.g., Hyperladder I (*see* **Subheading 3.1.1., step 8**).
3. Ligate the PCR product to the TOPO vector. Mix fresh PCR product (up to 4 μL), salt solution (1 μL), and TOPO vector (1 μL). Make up to a final volume of 6 μL with sterile water. Incubate at room temperature (22–23°C) for 5 min. For optimal efficiency, use a molar ratio of PCR product to TOPO vector of between 0.5:1 and 2:1.
4. Transform competent *E. coli* (50 μL) with 2 μL of the TOPO cloning reaction mixture (from **step 3**). Spread transformed cells on an LB-agar plate containing kanamycin (50 μg/mL), and incubate at 37°C overnight.
5. Pick 5–10 colonies (*see* **Note 2**), and culture each at 37°C overnight in LB medium containing kanamycin (50 μg/mL).
6. Isolate plasmid DNA by a standard method.
7. To confirm the presence, size, and orientation of the insert, analyze plasmid DNA by restriction digestion with an enzyme that cuts asymmetrically within the insert, together with *Not*I or *Asc*I, which cut either side of the cloning site (*see* **Fig. 2**).
8. Select a plasmid that has an insert of the correct size and orientation. Sequence the insert, to ensure that no errors have been introduced during the PCR or cloning procedures and that the cDNA/vector junctions are correct (*see* **Note 3**).
9. Restreak a colony (from **step 5**) that contains the correct insert and prepare a glycerol stock. Store at –80°C.
10. Store a stock of plasmid DNA at –20°C.

### *3.2.2. Site-Specific Recombination In Vitro*

Various BaculoDirect DNAs are available from Invitrogen. They were constructed by homologous recombination between WT AcMNV DNA and a transfer plasmid containing a Gateway cassette. After recombination, the Gateway cassette replaces the native polyhedrin gene. BaculoDirect C-term linear DNA (**Fig. 3B**) enables the cDNA of interest to be expressed as a native protein or as a C-terminal fusion with a V5 epitope and a 6X His tag. BaculoDirect N-term and BaculoDirect secreted DNAs enable the cDNA to be expressed with an N-terminal 6X His tag and V5 epitope, joined to the protein by a TEV protease recognition site. In the Secreted version the N-terminal 6x his tag is preceded by a honeybee melattin secretin signal, which enables the expressed protein to be secreted from the insect cells.

The cDNA is transferred from the entry vector clone (*see* **Subheading 3.2.1., step 10**) to the BaculoDirect linear DNA by recombination between specific *att* sites, catalyzed by LR Clonase™. To reduce the possibility of contamination, perform **steps 1–3** of the following protocol in a sterile laminar flow hood.

1. To a 1.5-mL microcentrifuge tube, add 1 to 2 μL (100–300 ng) of entry clone plasmid DNA (from **Subheading 3.2.1., step 10**), 10 μL (300 ng) of BaculoDirect linear DNA, and 4 μL of 5X LR Clonase reaction buffer. Make up to a final volume of 16 μL with TE buffer, pH 8.0.
2. Thaw the LR Clonase enzyme mix on ice for approx 2 min. Vortex briefly (twice for 2 s each time).
3. To each sample (*see* **step 1**), add 4 μL of Clonase enzyme mix. Do not vortex or pipet up and down, because this will shear the baculovirus DNA.
4. Incubate at 25°C for 1 h.
5. Add 2 μL of proteinase K solution. Incubate at 37°C for 10 min.
6. Transfect *Sf*9 insect cells with the LR recombination reaction mix (*see* **Subheading 3.5.**).

## *3.3. Propagation of Sf9 Insect Cells*

Cells can be grown as a monolayer or in shaking culture in the presence or absence of FCS, in a nonhumidified incubator at 27°C. Cells are propagated in SFM containing gentamicin (10 μg/mL). If serum is used, the concentration of gentamicin is increased to 50 μg/mL.

Subculture the cells when they reach mid-log phase (cell density of $2 \times 10^6$/mL). During routine subculturing, seed the cells at a density of $3 \times 10^5$ cells/mL.

### *3.3.1. Adaptation to Shaking Culture*

*Sf*9 cells grown in monolayer attach tightly to the surface of tissue culture vessels. The mechanical force needed to dislodge the cells during routine passaging damages them, resulting in a loss of viability. Growth of cells in shaking cultures increases their viability to >99%.

The adaptation of cells to suspension culture takes approx 2 to 3 wk and requires that cells be counted daily to monitor their viability.

1. Culture cells in SFM containing 10% (v/v) FCS and gentamicin (50 μg/mL) as monolayers in T175 flasks until confluent.

2. Dislodge the cells from the surface of the plastic vessel by tapping the flask quite vigorously and repeatedly against the palm of the hand. Alternatively, cell scrapers may be used.
3. Seed the cells in a 125-mL disposable Erlenmeyer flask at a density of $5 \times 10^5$ cells/mL. The total volume must not exceed 30 mL. If a larger culture is required, use a 250-mL disposable Erlenmeyer flask and a culture volume not exceeding 50 mL (*see* **Note 4**). Loosen the caps of the flasks slightly, to obtain aeration.
4. Shake the cells at an initial speed of 100 rpm at 27°C in a shaking incubator.
5. Count the cells daily and subculture when the density reaches $1–2 \times 10^6$ cells/mL. To subculture, seed the cells at a density of $5 \times 10^5$ cells/mL.
6. Increase the speed of shaking in increments of 5 rpm after each subculturing step until a speed of 135 rpm is reached without any loss in cell viability (*see* **Notes 5** and **6**).
7. Cryopreserve stock cultures of shaking-adapted cells (*see* **Subheading 3.4.**).

### 3.3.2. Adaptation to Serum-Free Conditions

The exclusion of serum is cheaper and allows standardization of the conditions in which cells are grown. In our laboratory, we routinely culture cells in Sf-900II SFM containing gentamicin (10 µg/mL) (*see* **Note 7**).

1. Dilute 100 µL of cells (*see* **Subheading 3.3.1., step 6**) with 900 µL of PBS. Add 100 µL of Trypan blue to 1 mL of diluted cells and, using a hemocytometer, count the number of viable cells. Dead cells are blue.
2. Seed the cells (from **Subheading 3.3.1., step 6**) at a density of $3 \times 10^5$ viable cells/mL in SFM containing 10% (v/v) FCS and gentamicin (50 µg/mL).
3. At the next passage, when the cells have reached a density of $2 \times 10^6$/mL, approx 3–4 d later, halve the serum concentration to 5% (v/v). Continue to halve the serum concentration at every passage until the cell viability remains constant at >98%. The serum concentration at which this usually occurs is 2.5%. The cells can now be grown in SFM containing gentamicin (10 µg/mL).
4. Cryopreserve stock cultures of cells adapted to SFM (*see* **Subheading 3.4.**).

### 3.3.3. General Maintenance

1. Passage the cells every 3–4 d or when they reach a density of $2 \times 10^6$ cells/mL.
2. Seed the cells at a density of $3 \times 10^5$ cells/mL.

## 3.4. Cryopreservation of Sf9 Cells

1. Use cells with a viability of >98% in their mid-log phase ($2 \times 10^6$ cells/mL).
2. Centrifuge the cells at 100*g* for 5 min and resuspend in the appropriate volume of cryopreservation medium. The density of the cells to be frozen should be $1–2 \times 10^7$ cells/mL. The cryopreservation medium depends on whether cells have been cultured in SFM or serum-containing medium (*see* **Subheading 2.4., steps 2** and **3**).
3. Aliquot the cells into cryovials and store on ice for 30 min.
4. Place the cryovials at –70°C for 3–4 h and then transfer to liquid nitrogen. Cells should not be kept at –70°C for more than 24 h.
5. Recover the cells by standard methods; that is, thaw the cells rapidly in a 37°C water bath and place the entire contents of a vial into prewarmed medium (*see* **Note 8**).

### 3.5. Transfection of *Sf*9 Cells With Recombinant Bacmid or Baculoviral DNA (see Note 9)

1. Seed cells at a density of $9 \times 10^5$ cells/35-mm dish (*see* **Note 10**) in 2 mL of SFM containing gentamicin. Only cells with a viability of >98% should be used; otherwise transfection efficiency is reduced.
2. Allow the cells to attach at 27°C for about 1 h. During this time prepare solutions A and B (*see* **Subheading 2.5., steps 6** and **7**).
3. Combine solutions A and B and incubate for 15–45 min at room temperature.
4. Remove the culture medium and wash the cells once with 2 mL of SFM without gentamicin.
5. Add SFM without gentamicin to the mix of solutions A and B to give a final volume of 1 mL. Gently overlay the cells with this solution, and incubate at 27°C for 5 h in a sandwich box containing damp tissues (humidified box).
6. Remove the transfection mixture after 5 h and replace with 2 mL of SFM containing gentamicin (*see* **Note 9**). Incubate at 27°C for 72 h in a humidified box.
7. Harvest the virus (supernatant) by transferring the medium in the dishes to a tube and centrifuging at 100*g* at 4°C for 10 min. The virus is present in the supernatant. Initial viral titers range from $2 \times 10^5$ to $4 \times 10^5$ plaque-forming units (PFU)/mL. To determine viral titer, perform a viral plaque assay (*see* **Subheading 3.7.**).
8. Store the virus at 4°C in the dark.

### 3.6. Viral Amplification

The stock virus is usually not sufficient for large-scale expression of proteins and must be amplified.

1. To amplify the virus, infect a monolayer culture of *Sf*9 cells (seeded at a density of $2 \times 10^6$ cells/mL) (*see* **Notes 11** and **12**) at a multiplicity of infection (MOI) of 0.01 to 0.1 PFU/cell.
2. To calculate the amount of inoculum required, the following formula may be used:

$$\text{Inoculum required (mL)} = \frac{\text{desired MOI (PFU/cell)} \times \text{(total number of cells)}}{\text{Titer of viral inoculum (PFU/mL)}}$$

3. Harvest the virus 72 h postinfection (*see* **Subheading 3.5., step 7** and **Note 13**).

### 3.7. Viral Plaque Assay

The viral plaque assay is simple but requires one to work quickly and accurately.

1. Plate 2 mL of a mid-log phase culture of *Sf*9 cells at a density of $5 \times 10^5$ cells/mL per well of a six-well plate. Plate sufficient wells to carry out each assay in duplicate.
2. Allow the cells to attach for 1 h at room temperature (*see* **Note 14**). While the cells attach, serially dilute ($10^{-1}$–$10^{-8}$) the virus stock to be titered (*see* **Subheading 3.5., step 7**, or **Subheading 3.6., step 3**) in SFM containing gentamicin. Prepare 5 mL of each dilution.
3. After cell attachment, aspirate the medium from the wells (*see* **Note 15**).
4. Carry out each assay in duplicate. Add 1 mL of the diluted virus ($10^{-3}$–$10^{-8}$) to the cells in the appropriate well. Incubate at room temperature for 1 h.
5. Melt 4% agarose in a 70°C water bath (*see* **Note 16**).
6. Just before use, dilute the agarose in SFM containing gentamicin to give a final concentration of 1%. Make up sufficient of this solution for 2 mL/well (*see* **Note 17**). Keep this solution at 37°C.
7. After the diluted virus has been incubated with the cells for 1 h (*see* **step 4**), remove the

virus by gently aspirating it. Then quickly add the agarose overlay to the cells. To do this, place a pipet against the side of the well and gently allow a steady stream of agarose to flow into the well. This procedure must be done rapidly to avoid setting of the agarose.

8. Allow the overlay to harden for 10–20 min. Place the plates in a sealed box containing damp tissues. Incubate at 27°C for 4–5 d.
9. After about 4–5 d neutral red can be added to the wells. Melt 4% agarose and add it to the neutral red solution in SFM containing gentamicin to give a final concentration of 1% agarose.
10. Remove the plates from the incubator and overlay each well with 1 mL of neutral red solution (as described in **step 7**). Allow the agarose to set (*see* **step 8**). Return the plates to the incubator.
11. Check for plaques regularly. Plaques show up as clear areas in a lawn of red cells and can be observed and counted by eye.
12. Calculate the viral titer using the following equation:

$$\text{PFU/mL} = \frac{\text{no. of plaques (PFU)}}{\text{Dilution factor} \times \text{mL of inoculum}}$$

### 3.8. Analysis of Viral DNA

#### 3.8.1 Testing of Virus for Correct Sequence Insertion

To ensure that the virus contains the appropriate cDNA, perform PCR analyses using M13F and M13R primers or M13F and a cDNA sequence-specific primer. To do this, the viral DNA must first be extracted.

1. Combine 89 µL of lysis buffer, 10 µL of amplified virus stock, and 1 µL of proteinase K (6 mg/mL) and incubate at 60°C for 1 h.
2. Inactivate the proteinase K by incubating at 95°C for 10 min, and cool the solution to room temperature. Place on ice.
3. Use 5 µL of the lysed virus directly in a PCR.
4. For PCR amplification of bacmid DNA, use conditions for long-range PCR (*see* **Note 18**).

#### 3.8.2.Testing for Viral Infectivity

If it is suspected that the virus has lost the ability to infect the cells, test the DNA from infected cells. Only DNA isolated from successfully infected cells will amplify in a PCR using M13 primers.

1. Culture cells in 35-mm dishes ($2 \times 10^6$ cells/dish) in 2 mL of SFM containing gentamicin.
2. Incubate the cells at 27°C for 1 h, to allow for attachment.
3. Add the amount of viral stock required to give an MOI of 5–10 PFU/cell (*see* **Subheading 3.6., step 2**) to each dish and incubate at 27°C for 72 h.
4. Aspirate the medium and replace it with 2 mL of PBS. Remove the PBS and add 2 mL of fresh PBS. Dislodge the cells from the plastic surface of the tissue culture dish by pipetting the liquid up and down to slough the cells from the surface of the dish.
5. Centrifuge the cell suspension at 1000*g* for 5 min at room temperature and remove the supernatant.
6. Resuspend the cells in 250 µL of TlowE buffer. Add 250 µL of cell lysis buffer and mix

thoroughly by inversion.

7. Add 12.5 µL of proteinase K (10 mg/mL) and 2.5 µL of RNase A (10 mg/mL). Incubate the reaction mixture at 37°C for 30 min.
8. Extract with phenol/chloroform (1:1 [v/v]) (*see* **Note 19**).
9. Transfer the upper layer to a clean microcentrifuge tube. Precipitate DNA by adding 50 µL of 3 *M* sodium acetate, pH 5.2, and 1 mL of ethanol. A white stringy DNA precipitate should be observed.
10. Centrifuge at 14,000*g* in a benchtop centrifuge, for 5 min at room temperature.
11. Remove the supernatant, add 150 µL of 70% ethanol, and centrifuge as in **step 10**.
12. Remove the supernatant and air-dry the DNA pellet. Resuspend in 100 µL of TlowE buffer.
13. For PCRs dilute the DNA 1:100 in water. Amplify using long-range PCR conditions.

## 3.9. Expression and Analysis of Recombinant Protein

### 3.9.1. Infection of Cells

Optimal infection conditions for the expression of FMOs in insect cells can vary among batches of virus. Conditions must be determined empirically for each viral batch. The time course for optimal expression of FMOs tends to be 72 h. Longer periods lead to cell death and protein degradation. Although the proteins can be expressed in monolayer cultures, larger yields are obtained from suspension cultures.

1. Infect a shaker culture (seeded at a density of $2 \times 10^6$ cells/mL) at an MOI of 5–10 PFU/cell with the appropriate virus.
2. At 72 h postinfection, recover the cells by centrifuging at 1000*g* for 10 min at 4°C. Discard the supernatant. The cell pellet can be stored at –70°C until use.

### 3.9.2. Isolation of Microsomal Membranes

Insect cells do not require harsh conditions to be lysed. We have found that sonication, three 10-s bursts on ice, leads to cell lysis with no protein degradation. Because sonicators vary, test the efficiency of lysis using trypan blue exclusion staining (*see* **Subheading 3.3.2., step 1**). Approximately 98% of the cells should be lysed before the next steps are carried out.

1. Sonication is carried out in sonication buffer containing PMSF. The volume of buffer required is approximately three times the volume of the cell pellet.
2. After sonication, centrifuge the lysed cell suspension at 1000*g* for 10 min at 4°C, to remove nuclei and cellular debris. Then centrifuge the resultant supernatant at 100,000*g* for 1 h at 4°C, to obtain a crude preparation of microsomal membranes. The pellet will contain both smooth and rough ER. If desired, standard ultracentrifugation methods can be used to purify further the microsomal membranes ***(15)***.

### 3.9.3. Analyses of Protein Expression

1. Determine the protein concentration by standard methods.
2. Electrophorese 10 µg of total microsomal proteins on SDS-polyacrylamide gels and stain with Coomassie blue. The expressed protein should be clearly visible with this stain and comprises about 20–30% of the total microsomal protein (*see* **Fig. 4A**).
3. To confirm that the correct protein has been expressed use Western blotting.

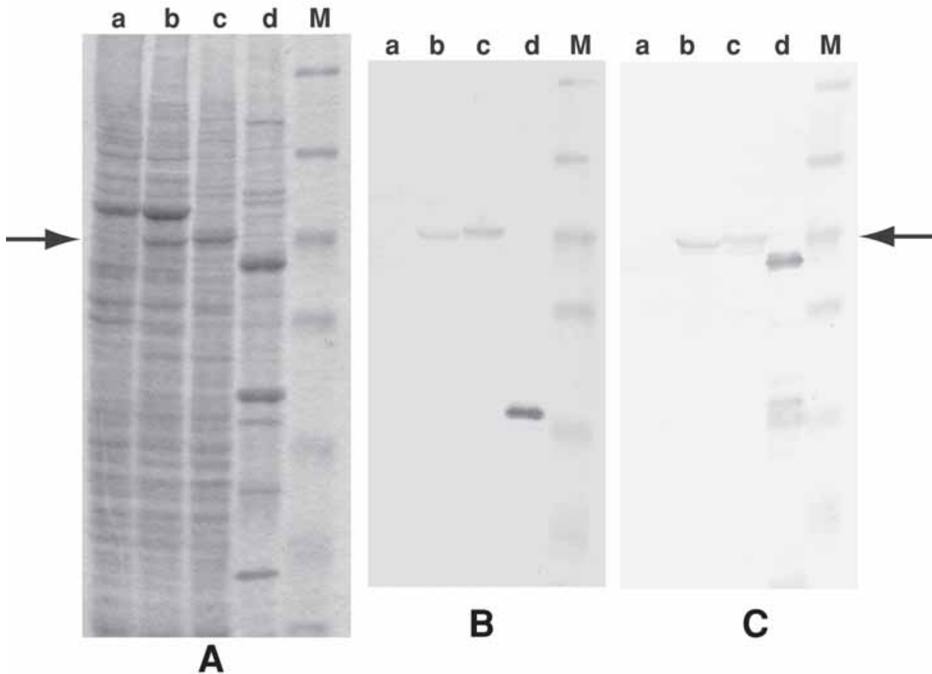


Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of microsomal membrane proteins isolated from baculoviral-infected *Sf*9 cells. Microsomal membrane proteins (20 μg) were isolated from *Sf*9 cells infected with WT baculovirus (lane a), or with a recombinant baculovirus encoding His-tagged Flavin-containing monooxygenase (FMO) 4 of mouse (lane b) or human (lane c). Lane d contains, as a positive control, either **(A,C)** 0.5 μg of membrane proteins isolated from bacteria expressing human FMO3 (gift of R. M. Philpot), or **(B)** 0.7 μg of his-tagged Internalin C (~32 kDa) (gift of R. W. Pickersgill). Lane M contains 10 μL of prestained broad-range protein marker (Bio-Rad). After electrophoresis through a 10% polyacrylamide gel containing SDS, proteins were **(A)** stained with Coomassie blue, or analyzed by **(B)** Western blotting with an anti-His antibody (1:1000 dilution) or with **(C)** an antirabbit FMO3 antibody (1:3000 dilution), which crossreacts with FMO4 (gift of R. M. Philpot). Arrows indicate the position of heterologously expressed FMO4s (~60 kDa).

4. Probe the blots with Anti-His antibodies (if the protein is His-tagged) or with the appropriate protein-specific antibody (*see* **Fig. 4B,C**). Assays to determine the activity of the protein can then be carried out.

## 4. Notes

1. To confirm that bacmid clones contain the correct sequence, carry out a PCR analysis using M13F and M13R primers. M13 sequences are present in the bacmid, flanking the cDNA insert (*see* **Fig. 5**). Alternatively, use an M13F primer and a cDNA sequence–specific primer. DNA products obtained from a typical PCR are shown in **Fig. 5B**.

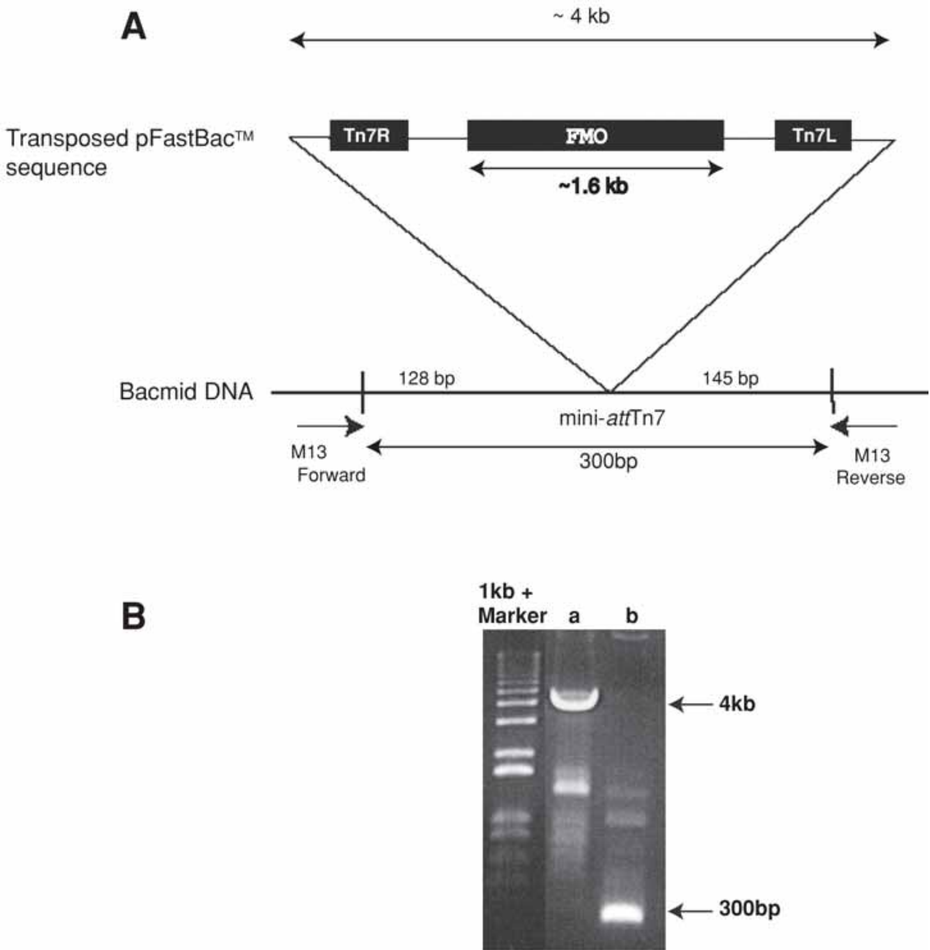


Fig. 5. **(A)** Diagrammatic representation of sequences of a pFastBac plasmid containing flavin-containing monooxygenase (FMO) cDNA, after transposition into the bacmid. Sizes of the FMO cDNA (~1.6 kb) and of the complete transposed sequence, flanked by the Tn7 elements (~4 kb), are indicated, as are the priming sites of M13 forward and reverse primers. The diagram is not to scale. **(B)** Agarose gel electrophoresis of bacmid DNA amplified by PCR using M13F and M13R primers. Amplification of DNA from a bacmid that has been successfully transposed with the FMO cDNA yields a 4-kb fragment (lane a), whereas amplification from a bacmid that contains no insert yields a 300-bp fragment (lane b).

2. There is no blue-white assay to identify recombinants. Plasmids must be isolated and analyzed to establish the presence of an insert.
3. Sequencing reactions can be primed with M13 forward and reverse primers (binding sites for these primers are present in the vector on either side of the cloning site). Primers complementary to internal cDNA sequences can also be used.

4. Culturing cells in a volume larger than 50 mL/250-mL flask tends to result in decreased cell viability owing to poor aeration.
5. If the cell viability decreases during this procedure, reduce the speed until the viability is >80%. We have found that in shaking cultures the cell viability tends to be maintained at >99%, which is ideal for subsequent procedures.
6. Only subculture and use cells when they are in mid-log phase. They must not be allowed to reach stationary phase (for cells in SF-900 II, with or without FCS, this corresponds to a density of $5 \times 10^6$ cells/mL).
7. Cells grown in serum-containing medium cannot be transferred directly to SFM, but must be gradually adapted to SFM.
8. When thawing shaker culture-adapted cells, first plate as a monolayer and then transfer into shaking culture. Monitor viability before use. Cells do not have to be readapted to shaker culture.
9. Methods for transfection of *Sf*9 cells with recombinant bacmid or baculoviral DNA generated as described in **Subheadings 3.1.** and **3.2.**, respectively, are identical, except for the inclusion of ganciclovir (100 µg/mL) in the medium (*see* **Subheading 3.5., step 6**).
10. For convenience, when dealing with a single type of recombinant virus, we use individual dishes rather than six-well plates, but six-well plates may be used when more than one type of recombinant virus is to be produced.
11. We have found that better amplification of the virus occurs when the cells are grown in a monolayer, although suspension cultures may be used.
12. For amplification of virus obtained from cells transfected with the products of the recombination reaction (*see* **Subheading 3.2.2.**), ganciclovir (100 µg/mL) should be included in the medium. For subsequent amplifications, ganciclovir is not necessary.
13. A viral titer can be determined at this stage, although the amplification procedure tends to lead to about a 100-fold amplification of the virus. If the viral titer is still low, further amplifications of the amplified virus can be carried out. Up to three rounds of amplification can be carried out without any deleterious effects on the virus. However, further amplification is not recommended because it will decrease viral infectivity.
14. To obtain an even monolayer, a platform shaker set at the lowest speed works best.
15. To keep cells from drying out, the use of a vacuum aspirator is a convenient and quick way of aspirating the medium.
16. We purchase the agarose as a 4% gel. Although one can make up the solution, using solid agarose, the drawback of the homemade solution is that sometimes, depending on the agarose used, contaminants may affect the growth of the cells or kill them.
17. We have used the more concentrated medium available from Invitrogen (i.e., 1.3 × Sf-900 II) but found that there was no difference in cell growth or viability when compared with the use of normal medium.
18. In our experience, BIO-X-ACT (Bioline) gives consistent results.
19. The appropriate precautions should be taken when using phenol/chloroform. All extractions should be done in a fume hood while adhering to appropriate safety precautions.

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# 6

# Cytochrome P450 Reconstitution Systems

## Hiroshi Yamazaki and Tsutomu Shimada

**Summary**

Human liver microsomes contain multiple forms of cytochrome P450 (CYP or P450) that catalyze oxidation of a number of xenobiotic and endobiotic chemicals. Individual P450 forms have unique, but overlapping, substrate specificities. It is necessary to determine which P450s play more important roles in the oxidation of these chemicals. A good way of studying the roles of P450s in the metabolism of these chemicals is to reconstitute the activities by mixing purified P450s and nicotinamide adenine dinucleotide phosphate–cytochrome P450 reductase in the membranes of phospholipid vesicles. However, our studies have suggested that the conditions for reconstitution of activities vary depending on the P450 enzymes used. For example, some reactions catalyzed by P450s require cytochrome-$b_5$ and a particular phospholipid environment for exerting their full catalytic activities. In this chapter, we describe optimal conditions that have been determined in our laboratories for the reconstitution of drug oxidation activities catalyzed by purified human CYP1A2, 2C9, 2E1, and 3A4.

**Key Words:** Reconstitution; CYP1A2; CYP2C9; CYP2E1; CYP3A4; nicotinamide adenine dinucleotide phosphate–cytochrome P450 reductase; lipid mixtures; cytochrome-$b_5$; ionic strength; apocytochrome $b_5$.

## 1. Introduction

Multiple forms of cytochrome P450 (P450 or CYP) exist in liver microsomes. These P450 forms have been shown to play important roles in the oxidation of structurally diverse xenobiotic chemicals, such as drugs, toxic chemicals, and carcinogens, as well as endobiotic chemicals, including steroids, fatty acids, fat-soluble vitamins, and prostaglandins *(1)*. Major P450s in human liver microsomes identified to date include CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 (and 3A7 in fetal liver) *(2)*. CYP1A1 and 1B1 have also been reported to be involved in the oxidation of drugs, toxic chemicals, and carcinogens in extrahepatic tissues *(3)*. There are large interindividual variations in the amount of each of these P450s, and these variations

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are considered to be one of the major factors in contributing to differences in susceptibilities of humans toward actions and toxicities of drugs, toxic chemicals, and carcinogens ***(2)***. To investigate the consequences for enzyme activity of genetic polymorphisms of individual P450s in humans, studies have been conducted using purified P450s isolated from heterologous organisms in which native and modified human P450 cDNAs have been introduced ***(4)***.

The conditions required to achieve maximal catalytic activities in reconstituted monooxygenase systems containing purified P450, nicotinamide adenine dinucleotide phosphate (NADPH)–cytochrome P450 reductase, and synthetic phospholipid vesicles have been shown to depend on the particular P450 used ***(5)***. Catalytic activities of CYP3A4 are higher when high concentrations of phosphate buffer are used. On the other hand, CYP2C9 is shown to have higher catalytic activities when low concentrations of phosphate buffer are used (*see* **Note 1**) ***(6)***. In some cases, particularly when CYP3As are used for reconstitution, several factors, including a particular lipid environment, cytochrome-$b_5$, divalent metal ions such as $Mg^{2+}$, and reduced glutathione (GSH), have been reported to be important for maximal catalytic activities ***(7–9)***. Other human P450s that have been shown to require cytochrome-$b_5$ and a particular lipid environment for optimal reconstitution include CYP2E1 and CYP2C9 and, possibly, CYP2C19, but the mechanisms underlying stimulation by cytochrome-$b_5$ have been reported to differ depending on the P450s examined ***(10,11)*** (*see* **Note 2**).

In this chapter, we describe the detailed conditions for reconstitution of drug oxidation activities catalyzed by human P450s, including

1. 7-Ethoxycoumarin *O*-deethylation catalyzed by CYP1A2, which does not require cytochrome-$b_5$ and a particular lipid environment for maximal catalytic activities.
2. *S*-Warfarin 7-hydroxylation catalyzed by CYP2C9, which requires cytochrome-$b_5$, a particular lipid environment, and a relatively low concentration of buffer ***(6)***.
3. Chlorzoxazone 6-hydroxylation catalyzed by CYP2E1, which requires cytochrome-$b_5$ and a particular lipid environment but does not require some other factors, such as $Mg^{2+}$ and GSH ***(11)***.
4. Testosterone 6β-hydroxylation catalyzed by CYP3A4, which requires cytochrome-$b_5$, a particular lipid environment, a high concentration of buffer, $Mg^{2+}$, and/or GSH, for improving catalytic activities ***(6,10)***.

## 2. Materials

### *2.1. Solutions*

1. 1.0 *M* potassium phosphate buffer, pH. 7.4.
2. 1.0 *M* potassium *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonate) (HEPES) buffer, pH 7.4.
3. TGE buffer: 10 m*M* Tris-HCl (pH 7.4) containing 20% (v/v) glycerol and 1 m*M* EDTA.
4. L-α-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC): make a solution of 1 mg of DLPC/mL of 10 m*M* potassium phosphate buffer, pH 7.4, and sonicate it with an ultrasonic disruptor (four cycles for 20 s each) at 4°C before use. The stock solution can be kept at –20°C for more than 1 mo but needs to be sonicated again before use (*see* **Note 3**).
5. Phospholipid mixture (DLPC, L-α-dioleoyl-*sn*-glycero-3-phosphocholine, and L-α-phosphatidyl-L-serine): make separate solutions of each of the three phospholipids (1 mg/mL

of 10 m*M* potassium phosphate buffer, pH 7.4), sonicate as previously described (*see* **item 4**), and then mix these solutions and sonicate again. The stock solution can be kept at –20°C as described (in **item 4** *see* **Notes 3** and **4**).

6. NADPH-generating system consisting of 5 m*M* $NADP^+$, 50 m*M* glucose-6-phosphate, and glucose-6-phosphate dehydrogenase (50 U/mL) (*see* **Note 5**).
7. 25 m*M* sodium cholate: dissolve 20 g of recrystallized cholic acid in 100 mL (20% [w/v]) of 0.05 *M* NaOH and adjust with 0.1 *M* HCl to pH 7.4 (cholic acid of commercial origin needs to be recrystallized from hot 50% ethanol, with the use of activated charcoal and celite to remove impurities). Dilute 20-fold with $H_2O$.
8. 150 m*M* GSH in 10 m*M* potassium phosphate buffer, pH 7.4.
9. 1.0 *M* $MgCl_2$.
10. 2 m*M* 7-ethoxycoumarin (Sigma, St. Louis, MO): dissolve 1.9 mg of 7-ethoxycoumarin in 1.0 mL of methanol or dimethyl sulfoxide (DMSO). Dilute fivefold with methanol or DMSO.
11. 10 m*M* *S*-warfarin (Ultra Fine Chemical, Manchester, UK): dissolve 3.1 mg of *S*-warfarin in 1.0 mL of methanol.
12. 50 m*M* chlorzoxazone (Sigma): dissolve 8.4 mg of chlorzoxazone in 1.0 mL of methanol.
13. 20 m*M* testosterone (Sigma): dissolve 5.8 mg of testosterone in 1.0 mL of methanol.
14. 10 μ*M* 7-hydroxycoumarin (Sigma): dissolve 1.6 mg of 7-hydroxycoumarin in 1 mL of methanol. Dilute 1000-fold with methanol.
15. 10 μ*M* 7-hydroxywarfarin (Ultra Fine Chemical): dissolve 3.2 mg of 7-hydroxywarfarin in 1.0 mL of methanol. Dilute 1000-fold with methanol.
16. 10 μ*M* 6-hydroxychlorzoxazone (Ultra Fine Chemical): dissolve 1.9 mg of 6-hydroxychlorzoxazone in 1.0 mL of methanol. Dilute 1000-fold with methanol.
17. 10 μ*M* 6β-hydroxytestosterone (Sigma): dissolve 3.0 mg of 6β-hydroxytestosterone in 1.0 mL of methanol. Dilute 1000-fold with methanol.
18. Trichloroacetic acid (10% [w/v]).
19. Perchloric acid (60% [w/v]).
20. Chloroform.
21. 30 m*M* sodium borate, pH 9.0.

## 2.2. Purified Enzymes

1. CYP1A2: This enzyme has been purified from human liver microsomes ***(12)***. Alternatively, recombinant human CYP1A2, purified from membranes of *Escherichia coli* into which modified CYP1A2 cDNA has been introduced ***(13)***, is commercially available (Oxford Biomedical Research, Oxford, MI; Invitrogen, Carlsbad, CA) (*see* **Note 6**).
2. CYP2C9: This enzyme has been purified from human liver microsomes ***(14)***. Purified CYP2C9 is commercially available (Research Diagnostics, Flanders, NJ). Alternatively, recombinant human CYP2C9, purified from membranes of *E. coli* into which modified CYP2C9 cDNA has been introduced ***(15)***, is commercially available (Oxford Biomedical Research and Invitrogen).
3. CYP2E1: This enzyme has been purified from human liver microsomes ***(16)***. Recombinant human CYP2E1, purified from membranes of *E. coli* into which modified CYP2E1 cDNA has been introduced ***(17)***, is available from Oxford Biomedical Research and Invitrogen.
4. CYP3A4: This enzyme has been purified from human liver microsomes ***(18)***. Purified CYP3A4 is commercially available (Research Diagnostics). Recombinant human CYP3A4, purified from membranes of *E. coli* into which modified CYP3A4 cDNA has been introduced ***(19)***, is also available from Oxford Biomedical Research and Invitrogen.

5. NADPH–cytochrome P450 reductase: This enzyme has been purified from liver microsomes of various animals including mice, rats, rabbits, and humans *(20–23)*. Because the use of this enzyme from different species results in no significant differences in drug-oxidizing activities in cytochrome P450 reconstitution systems, many investigators use the enzyme isolated from liver microsomes of phenobarbital-treated rabbits *(5,22)*. Native and recombinant human NADPH–cytochrome P450 reductase may be purchased from Research Diagnostics and Oxford Biomedical Research, respectively. Recombinant rat NADPH–cytochrome P450 reductase is available from Invitrogen (*see* **Note 7**).
6. Cytochrome-$b_5$ has been purified from liver microsomes of various species of animals including mice, rats, rabbits, and humans *(14,22–24)*. As in the case for NADPH–cytochrome P450 reductase, cytochrome-$b_5$ isolated from liver microsomes of phenobarbital-treated rabbits is very useful for reconstitution experiments. Native and recombinant cytochrome-$b_5$ are available from Research Diagnostics and Invitrogen, respectively (*see* **Note 8**).

## 3. Methods

### *3.1. Reconstitution of 7-Ethoxycoumarin O-Deethylation by CYP1A2*

The incubation mixture (final volume of 1.0 mL) consists of CYP1A2 (25 pmol), NADPH–cytochrome P450 reductase (50 pmol; usually twofold excess with respect to P450), and DLPC (20 μg) in 100 m*M* potassium phosphate buffer (pH 7.4), containing an NADPH-generating system (0.25 μmol of $NADP^+$, 2.5 μmol of glucose-6-phosphate, and 0.25 U of glucose-6-phosphate dehydrogenase) and 7-ethoxyresorufin (10 nmol).

1. Mix CYP1A2, NADPH–cytochrome P450 reductase, and DLPC. Incubate at 25°C for 5 min with shaking.
2. Add a solution of 105 μL of the NADPH-generating system (50 μL of 5 m*M* $NADP^+$, 50 μL of 50 m*M* glucose-6-phosphate, and 5 μL of glucose-6-phosphate dehydrogenase [50 U/mL]; 100 μL of 1.0 *M* potassium phosphate buffer, pH 7.4; and water (to give a final volume of 1.0 mL).
3. Add 5.0 μL of 2 m*M* 7-ethoxycoumarin. Incubate at 37°C for 5–10 min with vigorous shaking (approx 100 cycles/min).
4. Terminate the reaction by adding 50 μL of 10% $Cl_3CCOOH$ (w/v). (Terminate by adding 50 μL of 60% $HClO_4$ [w/v] for the direct high-performance liquid chromatography [HPLC] method *[25]*, instead of $Cl_3CCOOH$ for this spectrofluorometric method with metabolite extraction.)
5. Extract with 1.0 mL of $CH_2Cl_2$, and centrifuge at 900*g* for 5 min.
6. Re-extract the metabolites from aliquots (usually 0.5 mL) of the organic layer (lower layer) with 3.0 mL of 30 m*M* sodium borate (pH 9.0).
7. Measure the formation of 7-hydroxycoumarin fluorometrically in a spectrofluorometer at an excitation wavelength of 338 nm and an emission wavelength of 458 nm.

Alternatively, an HPLC method for a high-throughput system has also been developed *(25)*.

### *3.2. Reconstitution of S-Warfarin 7-Hydroxylation by CYP2C9*

The incubation mixture (final volume of 1.0 mL) consists of CYP2C9 (25 pmol), NADPH–cytochrome P450 reductase (50 pmol), cytochrome-$b_5$ (50 pmol), sodium

cholate (500 nmol), and the phospholipid mixture (20 µg) described in **Subheading 2.1., item 5**) in 50 m*M* potassium phosphate buffer (pH 7.4) containing the NADPH-generating system (*see* **Subheading 3.1.**) and S-warfarin (100 nmol).

1. Mix CYP2E1, NADPH–cytochrome P450 reductase, cytochrome-$b_5$, sodium cholate, and the phospholipid mixture (*see* **Subheading 2.1., item 5**). Incubate at 25°C for 5 min with shaking.
2. Add a solution of 105 µL of the NADPH-generating system (*see* **Subheading 3.1.**) 100 µL of 1.0 *M* potassium phosphate buffer, and water (to give a final volume of 1.0 mL).
3. Add 10 µL of 10 m*M* *S*-warfarin. Incubate at 37°C for 10–15 min with vigorous shaking (~100 cycles/min).
4. Terminate the reaction by adding 100 µL of 60% $HClO_4$ (w/v).
5. Centrifuge the mixtures at 900*g* for 5 min.
6. Determine product formation by HPLC with a $C_{18}$ 5-µm analytical column (4.6 × 150 mm; Kanto Chemical, Tokyo) eluted with a mixture of 36% $CH_3CN$ (v/v) containing 0.04% aqueous $H_3PO_4$ (w/v) at a flow rate of 1.5 mL/min. Perform fluoriometric detection at an excitation wavelength of 320 nm and an emission wavelength of 415 nm.

### 3.3. Reconstitution of Chlorzoxazone 6-Hydroxylation by CYP2E1

The incubation mixture (final volume of 1.0 mL) consists of CYP2E1 (25 pmol), NADPH-cytochrome P450 reductase (50 pmol), cytochrome-$b_5$ (50 pmol), sodium cholate (500 nmol), and the phospholipid mixture (20 µg) (*see* **Subheading 2.1., item 5**) in 100 m*M* potassium phosphate buffer (pH 7.4) containing the NADPH-generating system (*see* **Subheading 3.1.**), and chlorzoxazone (0.5 µmol).

1. Mix CYP2E1, NADPH–cytochrome P450 reductase, cytochrome-$b_5$, sodium cholate, and the phospholipid mixture (*see* **Subheading 2.1., item 5**).
2. Incubate at 25°C for 5 min with shaking.
3. Add a solution of 105 µL of the NADPH-generating system (*see* **Subheading 3.1.**), 100 µL of 1.0 *M* potassium phosphate buffer, and water (to give a final volume of 1.0 mL).
4. Add 10 µL of 50 m*M* chlorzoxazone (*see* **Note 9**) and incubate at 37°C for 10 min with vigorous shaking (~100 cycles/min).
5. Terminate the reaction by adding 100 µL of 43% $H_3PO_4$ (w/v) and 3.0 mL of $CH_2Cl_2$. Centrifuge the mixtures at 900*g* for 5 min and desiccate aliquots (1.5 mL) of the organic layer under an $N_2$ stream. Dissolve the residues in 100 µL of 27% $CH_3CN$ (v/v) in 0.5% $H_3PO_4$ (w/v).
6. Determine product formation by HPLC with a 4.6 × 150 mm Nucleosil octylsilyl ($C_8$) reverse-phase column (Chemco, Osaka) in a mobile phase consisting of a mixture of 27% $CH_3CN$ (v/v) in 0.5% aqueous $H_3PO_4$ (v/v) at a flow rate of 1.5 mL/min. Perform detection by ultraviolet (UV) absorbance at 287 nm.

### 3.4. Reconstitution of Testosterone Hydroxylation by CYP3A4

The incubation mixture (final volume of 1.0 mL) consists of CYP3A4 (25 pmol), NADPH–cytochrome P450 reductase (50 pmol), cytochrome-$b_5$ (50 pmol), sodium cholate (500 nmol), and the phospholipid mixture (20 µg) (*see* **Subheading 2.1., item 5**) in 50 m*M* potassium HEPES buffer (pH 7.4) containing the NADPH-generating system (*see* **Subheading 3.1.**), $MgCl_2$ (30 µmol), GSH (3 µmol), and testosterone (0.2 µmol) (*see* **Notes 10** and **11**).

1. Mix CYP3A4, NADPH–cytochrome P450 reductase, cytochrome-$b_5$, sodium cholate, and the phospholipid mixture (*see* **Subheading 2.1., item 5**).
2. Incubate at 25°C for 5 min with shaking.
3. Add a solution of 50 μL of 1.0 *M* potassium HEPES buffer, 105 μL of the NADPH-generating system (*see* **Subheading 3.1.**), 20 μL of 150 m*M* GSH, water (to give a volume of 1.0 mL), and 30 μL of 1.0 *M* $MgCl_2$ (*see* **Notes 11–14**).
4. Add 10 μL of 20 m*M* testosterone and incubate at 37°C for 10 min with vigorous shaking (~100 cycles/min).
5. Terminate the reaction by adding 100 μL of 1.0 *M* HCl containing 2.0 *M* NaCl (w/v) and 3.0 mL of $CH_2Cl_2$.
6. Centrifuge the mixtures at 900*g* for 5 min and desiccate aliquots (1.5 mL) of the organic layer under an $N_2$ stream.
7. Dissolve the residues in 100 μL of 64% (v/v) aqueous methanol.
8. Determine product formation by HPLC with a 4.6 × 150 mm $C_{18}$ reverse-phase column. Elute using a mixture of 64% (v/v) aqueous methanol at a flow rate of 1.5 mL/min and perform detection by UV absorbance at 240 nm ***(26)***.

## 4. Notes

1. Suitable concentrations of phosphate buffer in the reaction mixtures for each human P450 were different (**Fig. 1**). CYP2C9 preferred low concentrations of phosphate buffer for its maximal catalytic activities whereas CYP3A4 required high concentrations of phosphate buffer (with high ionic strength) or divalent metal ions, such as $Mg^{2+}$.
2. Human P450s that require cytochrome-$b_5$ for demonstration of maximal catalytic activities in reconstitution systems include CYP3A4, 3A5, 2E1, 2C19, 2C9, 2C8, 2B6, and 2A6 ***(10,11,14,27)***. However, the mechanisms underlying stimulation by cytochrome-$b_5$ have been shown to differ depending on the P450s used (**Fig. 2**). For example, apocytochrome-$b_5$ (devoid of heme) as well as native cytochrome-$b_5$ has been shown to be effective in stimulating testosterone 6β-hydroxylation and nifedipine oxidation by CYP3A4, whereas stimulation by apocytochrome-$b_5$ in reconstituted monooxygenase systems containing CYP2E1 did not occur ***(27–29)***. Apocytochrome-$b_5$ has improved the catalytic activities of CYP2A6, 2B6, 2C8, 2C9, 2C19, and 3A5 as well as those of CYP3A4 ***(27,28)***. Similar stimulation of CYP17A ***(30)*** and CYP4A7 ***(31)*** by apocytochrome-$b_5$ has been reported.
3. In some of the sonicated lipid stock solutions (DLPC, the phospholipid mixture, and the microsomal lipids), we include sodium cholate (final concentration of 25 m*M*). When the sodium cholate is added to the mixture, it does not need to be sonicated again.
4. The phospholipid mixture can be replaced with isolated lipids from liver microsomes of rats, rabbits, and humans ***(6)***.
5. The NADPH-generating system can be replaced with 1 m*M* NADPH (final concentration) when the incubation is done at 37°C within 10 min.
6. The purified P450s (suspended in TGE buffer after removing detergents used for the purification steps) are stable at –80°C for at least 1 yr. Concentrated enzymes (>5 μ*M* P450) are more stable and useful for the reconstitution experiments. When required, the concentrated P450 solution can be diluted with TGE buffer.
7. The purified NADPH–cytochrome P450 reductase (suspended in TGE buffer after removing detergents used for the purification steps) is stable at –80°C for at least 1 yr. Concentrated enzymes (>10 μ*M* NADPH–cytochrome P450 reductase) are more stable and useful for the reconstitution experiments. When required, the concentrated NADPH–cytochrome P450 reductase solution can be diluted with TGE buffer.

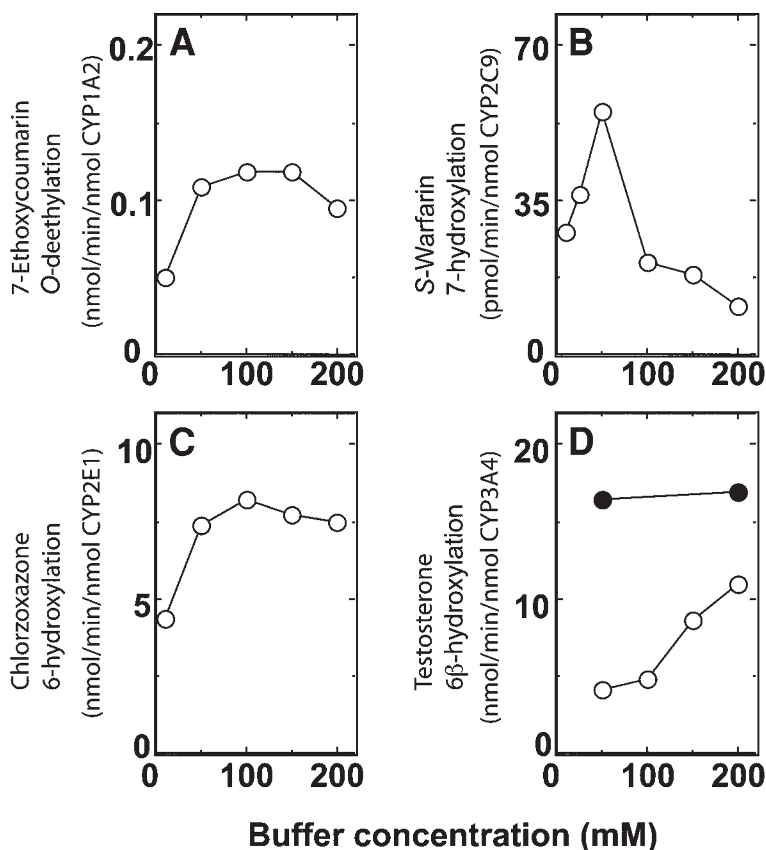


Fig. 1. **(A)** Effects of buffer concentrations on 7-ethoxycoumarin *O*-deethylation by CYP1A2, **(B)** *S*-warfarin 7-hydroxylation by CYP2C9, **(C)** chlorzoxazone 6-hydroxylation by CYP2E1, and **(D)** testosterone 6β-hydroxylation by CYP3A4 in reconstituted systems. The experimental conditions for each of the drug oxidations by P450s are described under **Subheading 3.** In the case of testosterone 6β-hydroxylation **(D)**, both potassium phosphate (○) and potassium HEPES plus $Mg^{2+}$ (●) buffers were examined, and in other cases **(A–C)** only the effects of potassium phosphate buffer were determined. Data represent means of duplicate or triplicate determinations.

8. Purified cytochrome-$b_5$ (suspended in TGE buffer or 100 m*M* potassium phosphate buffer, pH 7.4, after removing detergents used for the purification steps) is stable at –80°C for at least 1 yr. The concentrated protein (>10 µ*M* cytochrome-$b_5$) is more stable and useful for the reconstitution experiments. When required, the concentrated cytochrome-$b_5$ solution can be diluted with TGE buffer.
9. In the original method by Peter et al. ***(32)***, the stock solution of chlorzoxazone (50 m*M*) was dissolved in 60 m*M* potassium hydroxide (freshly prepared), to avoid the inclusion of organic solvents in the reaction mixture (organic solvents can interfere with CYP2E's activities).

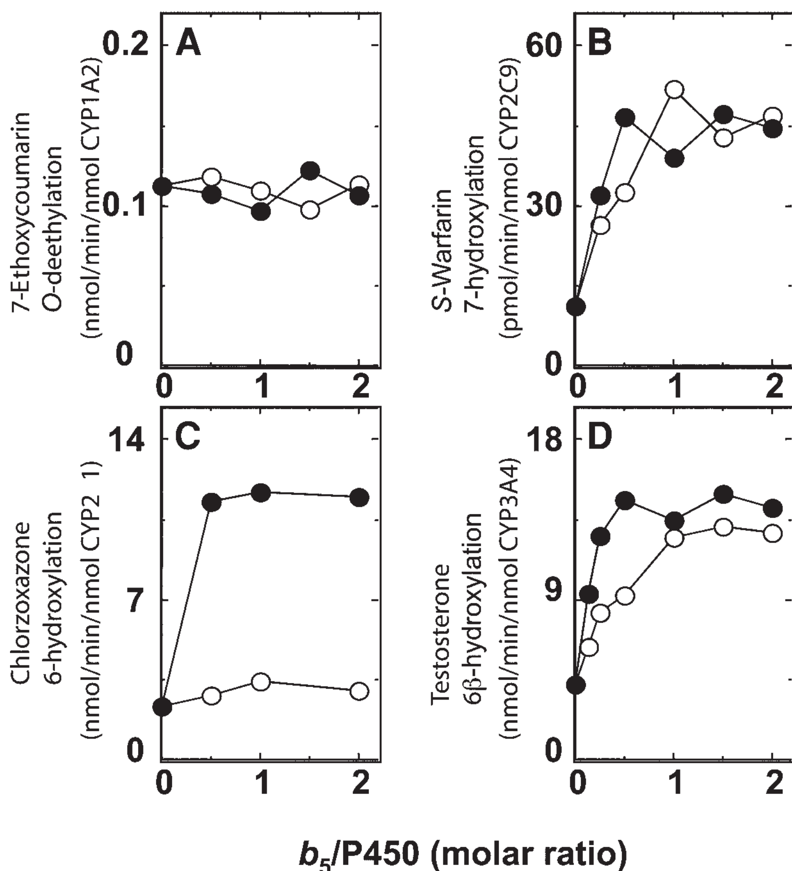


Fig. 2. Effects of concentrations of cytochrome-$b_5$ (●) and apocytochrome-$b_5$ (○) on **(A)** 7-ethoxycoumarin *O*-deethylation by CYP1A2, **(B)** *S*-warfarin 7-hydroxylation by CYP2C9, **(C)** chlorzoxazone 6-hydroxylation by CYP2E1, and **(D)** testosterone 6β-hydroxylation by CYP3A4 in reconstituted systems. The experimental conditions for each of the drug oxidations by P450s are described under **Subheading 3.** Data represent means of duplicate or triplicate determinations.

10. With testosterone 6β-hydroxylation and nifedipine oxidation reactions catalyzed by CYP3A4 in reconstituted systems, the following order of mixing was found to be optimal: CYP3A4, NADPH–cytochrome P450 reductase, cytochrome-$b_5$, sodium cholate, and a phospholipid mixture—these components are mixed at room temperature, followed by the addition of potassium HEPES buffer (pH 7.4) the NADPH-generating system, GSH (final concentration of 3.0 m*M*), $MgCl_2$ (final concentration of 30 m*M*), and substrate (testosterone or nifedipine) ***(28)***.
11. The stimulatory effects of cytochrome-$b_5$ in reconstituted monooxygenase systems containing CYP3A4 are not observed when *N*-demethylations of ethylmorphine and

benzphetamine are measured. The mechanisms by which cytochrome-$b_5$ stimulates some, but not other, CYP3A4-dependent reactions are still unclear ***(28)***.

12. $MgCl_2$ should be added just before the initiation of the reaction (with substrate), in order to avoid the formation of a precipitate.
13. The mechanisms by which $MgCl_2$ and GSH stimulate the testosterone 6β-hydroxylation activities by CYP3A4 in reconstituted systems are not known, although $MgCl_2$ and other divalent metal ions seem to stimulate reduction of cytochrome-$b_5$ ***(5)***. The effect of GSH is less pronounced when $Mg^{2+}$ is present.
14. When 50 m*M* potassium HEPES buffer (pH 7.4) was replaced with 50 m*M* potassium phosphate buffer (pH 7.4), the CYP3A4-dependent testosterone 6β-hydroxylation activities were also stimulated by $MgCl_2$. However, such stimulatory effects by $MgCl_2$ could not be determined when the potassium phosphate concentration was increased to 200 m*M*.

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# 7

# Catalytic Assays for Human Cytochrome P450

## *An Introduction*

**Thomas K. H. Chang and David J. Waxman**

**Summary**

Cytochrome P450 (P450) is a superfamily of individual monooxygenase enzymes that metabolize structurally diverse xenochemicals, including many clinically useful drugs and foreign chemicals widespread in the environment. P450 substrates that can be used to selectively monitor individual P450 enzymes or P450 subfamilies have been identified through studies using P450 enzyme-selective inhibitory antibodies and chemical inhibitors in conjunction with experiments utilizing individual cDNA-expressed P450 enzymes. This chapter describes P450 form-selective substrates that can be used to monitor the activities of human P450 enzymes CYP1A, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A, CYP4A11, and CYP7A1. Cautions that need to be exercised when using these substrates to probe for individual P450 activities in human liver and other tissues are discussed.

**Key Words:** Cytochrome P450; 7-ethoxyresorufin; 7-ethoxy-4-trifluoromethylcoumarin; *S*-mephobarbital; (*S*)-warfarin; (*S*)-mephenytoin; *p*-nitrophenol; chlorzoxazone; *O*-deethylation.

## 1. Introduction

Cytochrome P450 (P450) enzymes catalyze the biotransformation of a broad range of structurally diverse foreign chemicals and endogenous substances. Many P450 enzymes have been isolated and identified in liver and other tissues, including kidney, lung, intestines, and brain. Although these enzymes have a high degree of similarity in their amino acid sequences, many are subject to differential regulation and have distinct catalytic functions. With the development of P450 form-selective (or P450 subfamily-specific) inhibitory antibodies; the discovery of P450 enzyme-selective chemical inhibitors; and the commercial availability of catalytically active, individual P450 cDNA-expressed proteins, several substrates, including drugs and endogenous substances, have been identified as useful catalytic monitors for specific P450 enzymes

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or subfamilies (*see* **Table 1**). These catalytic monitors can serve as useful experimental tools in a variety of studies. These include studies designed to:

1. Identify individual P450s that catalyze the activation or detoxification of a particular compound.
2. Quantify the expression and catalytic activity of an individual P450 in tissue samples and in cultured cells.
3. Analyze enzyme kinetics for a particular P450-catalyzed reaction.
4. Elucidate the mechanism of interaction between xenobiotics and individual P450 enzymes.

Each of the substrate oxidation reactions provided in **Table 1** is suitable for measuring the catalytic activity of the corresponding cDNA-expressed P450 enzyme. However, errors can sometimes be made when interpreting activity data derived from cDNA-expressed enzyme studies. The fact that a cDNA-expressed human P450 can catalyze a particular substrate oxidation reaction does not necessarily mean that the corresponding enzyme will be an important catalyst in the same oxidation reaction in human tissues. For example, cDNA-expressed CYP1A1 is active in chlorzoxazone 6-hydroxylation ***(1)***. However, based on enzyme kinetic considerations and the tissue-specific distribution of human CYP1A1, this P450 is not expected to contribute significantly to chlorzoxazone 6-hydroxylation in human liver microsomes ***(2)***.

Caution should be exercised when using catalytic monitors as probes for human microsomal P450 activities for the following reasons. First, the validity of using a particular P450 substrate as a "diagnostic" catalytic monitor for a specific human microsomal P450 depends largely on the P450 form specificities of the anti-P450 antibodies and of the chemical inhibitors employed to define the P450 form specificity of the enzyme reaction. Second, potential crossreactivity with, or inhibition of, uncharacterized P450 enzymes or closely related P450 forms needs to be considered. For example, polyclonal antibodies that are immunoreactive with CYP2C9 typically crossreact with other CYP2C forms such as CYP2C8 and CYP2C19 ***(3)***, and the macrolide antibiotic triacetyloleandomycin is an effective inhibitor of many (perhaps all) CYP3A enzymes, including CYP3A4 and CYP3A5 ***(4)***. Thus, the catalytic contributions made by the individual CYP2C or the individual CYP3A enzymes cannot be distinguished using these analytical tools. Third, the definition of P450 form specificity in tissue microsomes will frequently depend on the substrate concentration used in the in vitro assay. For example, phenacetin *O*-deethylation activity is a marker for human hepatic microsomal CYP1A2 only if the assay is performed at low micromolar (e.g., 4 μ*M*) concentrations ***(5)***. This is because biphasic enzyme kinetics is observed in phenacetin *O*-deethylation catalyzed by human liver microsomes ***(6)***, with CYP1A2 corresponding to a low-$K_m$ form ***(7)*** and CYP2E1 to a high-$K_m$ form ***(8)***. Conducting the assay at higher substrate concentrations will result in loss of specificity of the catalytic monitor (*see* **Notes 14** and **20** for other examples). Fourth, the selectivity of a given P450 substrate probe can be tissue specific. For example, whereas CYP1A2 accounts for the majority of microsomal phenacetin *O*-deethylation activity in human liver, this P450 makes little or no contribution to phenacetin *O*-deethylation activity in human placenta microsomes ***(9)***. Finally, intersample differences exist in the extent to

**Table 1**
**Catalytic Monitors for Individual Human Cytochrome P450 Enzymes**

| Enzyme | Catalytic monitor | Assay method |
|---|---|---|
| CYP1A1/2 | 7-Ethoxyresorufin *O*-deethylation (***15***) (*see* **Note 1**) | Chapter 8 |
| | Phenacetin *O*-deethylation (***16,17***) (*see* **Note 2**) | **refs.** ***16*** and ***18*** |
| | Caffeine *N*3-demethylation (***7***) | **ref.** ***19*** |
| CYP1B1 | 7-Ethoxyresorufin *O*-deethylation (***20***) (*see* **Note 3**) | Chapter 8 |
| | 17β-Estradiol 4-hydroxylation (***20,21***) | **refs.** ***22*** and ***23*** |
| CYP2A6 | Coumarin 7-hydroxylation (***24,25***) (*see* **Note 4**) | Chapter 9; **ref.** ***18*** |
| CYP2B6 | 7-Ethoxy-4-trifluoromethylcoumarin *O*-deethylation (***26***) (*see* **Note 5**) | Chapter 10 |
| | *S*-mephenytoin *N*-demethylation (***27***) (*see* **Note 6**) | **ref.** ***27*** |
| | Bupropion hydroxylation (***28***) (*see* **Note 7**) | **refs.** ***18*** and ***28*** |
| | *S*-mephobarbital *N*-demethylation (***29***) (*see* **Note 8**) | **ref.** ***29*** |
| CYP2C8 | Paclitaxel 6α-hydroxylation (***30,31***) (*see* **Note 9)** | Chapter 11 |
| | Amodiaquine *N*-desethylation (***32***) (*see* **Note 10**) | **refs.** ***18*** and ***32*** |
| CYP2C9 | Diclofenac 4'-hydroxylation (***31,33***) (*see* **Note 11**) | Chapter 12; **ref.** ***18*** |
| | Tolbutamide 4'-hydroxylation (***31,34***) (*see* **Note 12**) | **refs.** ***18*** and ***35*** |
| | (*S*)-warfarin 7-hydroxylation (***36***) | **ref.** ***37*** |
| CYP2C19 | (*S*)-mephenytoin 4'-hydroxylation (***31,38***) (*see* **Note 13**) | Chapter 13; **ref.** ***18*** |
| CYP2D6 | Bufuralol 1'-hydroxylation (***39,40***) (*see* **Note 14**) | Chapter 14 |
| | Dextromethorphan *O*-demethylation (***40,41***) | **refs.** ***18,42***, and ***43*** |
| | Debrisoquine 4-hydroxylation (***16,44***) (*see* **Note 14**) | **ref.** ***42*** |
| CYP2E1 | *p*-Nitrophenol hydroxylation (***45***) (*see* **Note 15**) | Chapter 15 |
| | Chlorzoxazone 6-hydroxylation (***46,47***) (*see* **Note 16**) | **refs.** ***18,48***, and ***49*** |
| | Lauric acid 11-hydroxylation (***50***) | **ref.** ***50*** |
| | *N*-nitrosodimethylamine *N*-demethylation (***51,52***) | **ref.** ***53*** |
| CYP3A4/5 | Testosterone 6β-hydroxylation (***54,55***) (*see* **Note 17**) | Chapter 16; **ref.** ***18*** |
| | Midazolam 1'-hydroxylation (***56,57***) (*see* **Note 18**) | **refs.** ***18*** and ***57*** |
| | Nifedipine oxidation (***58,59***) | **ref**. ***58*** |
| CYP4A11 | Lauric acid 12-hydroxylation (***60***) (*see* **Note 19**) | Chapter 17 |
| CYP7A1 | Cholesterol 7α-hydroxylation (***61***) | Chapter 18 |

which a given P450 enzyme contributes to a catalytic activity in human liver microsomes. For example, immunoinhibition experiments with a monoclonal antibody (MAb) to CYP2E1 indicate that this P450 enzyme accounts for an average of 46% of the chlorzoxazone 6-hydroxylation activity in a panel of microsomes from 19 donor livers, but the degree of contribution to this activity by CYP2E1 in microsomes from a single donor varies from 20 to 80% ***(10)***. This presumably reflects interindividual differences in hepatic CYP2E1 protein expression ***(11)***.

Although certain compounds may be considered reasonably specific substrates for a particular P450 enzyme (or group of P450 enzymes in the same P450 subfamily), in the case of other P450 substrates, multiple enzymes may participate in their metabolism. Experimentally, this characteristic of a general (P450 form nonspecific) substrate provides a convenient approach to verifying that each P450 in a panel of recombinant P450 enzymes is indeed catalytically active ***(3,12–14)***. Substrates that are metabolized by many P450s may be useful in screening for potential interactions between a compound of interest and microsomal P450 enzymes.

Chapters 8–19 describe detailed experimental protocols using drugs and other xenobiotics as substrates for assaying individual P450 enzymes in the CYP1 (Chapter 8) and CYP2 (Chapters 9–15) families; endogenous substances as substrates for assaying CYP3A, CYP4A11, and CYP7A1 (Chapters 16–18); and a general substrate for assaying multiple P450 enzymes in the CYP1, CYP2, and CYP3 families (Chapter 19).

## 2. Notes

1. CYP1A2 accounts for a major fraction of 7-ethoxyresorufin *O*-deethylation activity in human liver microsomes ***(15)***. The role of hepatic CYP1A1 in this activity is minimal because this P450 is either absent or expressed at very low levels in liver ***(62)***.
2. Phenacetin *O*-deethylation activity is a marker for human hepatic microsomal CYP1A2 only if the assay is performed at a suitable substrate concentration (e.g., 4 µ*M*) ***(5)***. Conducting the assay at higher concentrations will result in loss of specificity of the substrate probe because of the contribution of a higher-$K_m$ P450 enzyme such as CYP2E1 ***(8)*** to the activity.
3. cDNA-expressed CYP1B1 is active in 7-ethoxyresorufin *O*-deethylation ***(20,63)***. However, CYP1B1 protein is undetectable in human liver ***(64)*** and, thus, does not contribute to 7-ethoxyresorufin *O*-deethylation activity in human liver microsomes.
4. Coumarin 7-hydroxylation activity is relatively specific for CYP2A6 in human liver microsomes: MAb to CYP2A6 inhibits >90% of the activity in human liver microsomes ***(25)***, and CYP2A6 is the sole catalyst of coumarin 7-hydroxylation in a panel of nine individual cDNA-expressed human P450 enzymes ***(65)***.
5. Owing to the simplicity of a fluorescence-based assay, the 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylation assay is commonly employed in studies with cDNA-expressed CYP2B6, such as those elucidating the mechanism of CYP2B6 inhibition ***(66)***, and in studies with intact cells expressing CYP2B6 ***(67)***. In studies with human liver microsomes, a method has been developed for estimating the CYP2B6 component of 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylation activity (*see* **ref. *26*** and Chapter 10) because several P450s, including CYP2B6, are catalytically active in 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylation ***(26)***.

6. (*S*)-mephenytoin *N*-demethylation is used as a catalytic monitor for CYP2B6 in human liver ***(68)***, but the enzyme assay must be conducted at a substrate concentration >1000 µ*M* in order to minimize the contribution of the lower-$K_m$ enzyme CYP2C9 ***(69)***.
7. Bupropion hydroxylation appears to be a suitable catalytic monitor of CYP2B6 in human liver microsomes. When enzyme activity is assayed at a substrate concentration of 500 µ*M*, CYP2B6 accounts for approx 90% of the activity in human liver microsomes ***(28)***, and CYP2E1 and CYP3A4 do not contribute to this activity ***(28,70)***.
8. (*S*)-mephobarbital *N*-demethylation is a candidate enzyme-selective marker for CYP2B6 in human liver microsomes. Experiments with cDNA-expressed enzymes indicate that this reaction is catalyzed by CYP2B6, but not CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E2, or CYP3A4 ***(29)***.
9. Among the 14 individual cDNA-expressed human P450 enzymes examined to date, only CYP2C8 is active in paclitaxel 6α-hydroxylation ***(30,71,72)***. The selectivity of this reaction for CYP2C8 has been confirmed in immunoinhibition experiments with MAb to CYP2C8 ***(31)***. Amodiaquine *N*-desethylation is a candidate marker for CYP2C8 in human liver microsomes. However, immunoinhibition experiments with CYP2C8-specific antibodies will need to be performed to determine the extent of contribution of hepatic CYP2C8 to this reaction.
10. CYP2C9 is estimated to contribute to 85–90% of diclofenac 4'-hydroxylation activity in human liver microsomes ***(31)***. Therefore, this reaction is a suitable catalytic monitor for CYP2C9 in human liver microsomes.
11. Although CYP2C19 is catalytically active in tolbutamide 4'-hydroxylation ***(73,74)***, CYP2C9 accounts for the major fraction (75–90%) of this activity in human liver microsomes ***(31)***. Accordingly, tolbutamide 4'-hydroxylation is frequently used as a marker for human hepatic CYP2C9 ***(68)***.
12. In a panel of 13 individual cDNA-expressed human P450 enzymes, only CYP2C9 and CYP2C19 are active in (*S*)-mephenytoin 4'-hydroxylation, but CYP2C19 is 20-fold more active than CYP2C9 ***(31)***. Based on immunoinhibition experiments, it is estimated that CYP2C19 contributes to 80–90% of this reaction in human liver microsomes, with the remainder owing to CYP2C9 ***(31)***.
13. When using bufuralol 1'-hydroxylation activity as a marker of CYP2D6 in human liver microsomes, it is necessary to conduct the assay at a suitable substrate concentration (e.g., 25 µ*M*) (*see* Chapter 14). Performing the assay at higher concentrations will result in loss of specificity of this substrate probe because of contribution of higher-$K_m$ forms such as CYP1A2 ***(75)***.
14. Experiments with individual cDNA-expressed human P450 enzymes have indicated that, in addition to CYP2D6, CYP1A1 is a highly active catalyst of debrisoquine 4-hydroxylation ***(44)***. However, CYP1A1 is unlikely to contribute substantively to this activity in human hepatic microsomes because of the absence or low expression levels of this P450 enzyme in the liver ***(62)***.
15. Exercise caution when using hepatic microsomal *p*-nitrophenol hydroxylation activity as a diagnostic catalytic monitor for CYP2E1 in human liver because of the potential contribution of CYP2A6 ***(76)*** and CYP3A ***(77)*** to this activity (*see* Chapter 15).
16. A recent report showed that human hepatic microsomal chlorzoxazone 6-hydroxylation activity reflects not only CYP2E1 but also CYP3A in human liver ***(78)***, indicating that this activity is not specific for hepatic CYP2E1 in vitro.
17. According to a recent immunoinhibition study with inhibitory antipeptide antibodies, hepatic CYP3A4 accounts for nearly all of the testosterone 6β-hydroxylation activity in

human hepatic microsomes, whereas hepatic microsomal CYP3A5 makes little or no contribution to this activity *(79)*.

18. Immunoinhibition experiments have established that CYP2B6 and CYP2C9 do not contribute to midazolam 1'-hydroxylation in human liver microsomes and that CYP3A4 accounts for most of this activity *(80)*.
19. When using hepatic microsomal lauric acid 12-hydroxylation activity as a marker of CYP4A11 in human liver, it is necessary to conduct the assay at a suitable substrate concentration (e.g., 100 μ*M*) (*see* Chapter 17). Performing the assay at higher concentrations will result in loss of specificity of this substrate probe because of the contribution of higher-$K_m$ forms, such as CYP2E1 *(81)*.

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# 8

## Enzymatic Analysis of cDNA-Expressed Human CYP1A1, CYP1A2, and CYP1B1 With 7-Ethoxyresorufin as Substrate

**Thomas K. H. Chang and David J. Waxman**

**Summary**

Cytochrome P450 (P450) enzymes belonging to the CYP1 family are highly inducible by polycyclic aromatic hydrocarbons and other environmental chemicals and play a major role in the metabolism of many foreign chemicals and endogenous substances. We describe a spectrofluorometric method for determining 7-ethoxyresorufin *O*-dealkylation catalyzed by CYP1A1, CYP1A2, and CYP1B1. The formation of the enzymatic product, resorufin, is monitored continuously by fluorescence using an excitation wavelength of 530 nm and an emission wavelength of 580 nm. This method can be applied to assay P450-catalyzed formation of resorufin from other alkoxyresorufins, such as 7-methoxyresorufin, 7-benzyloxyresorufin, and 7-pentoxyresorufin. It can also be used to assay 7-ethoxyresorufin *O*-dealkylation activity in isolated hepatocytes and cultured cells that express this P450 activity.

**Key Words:** Cytochrome P450; CYP1A1; CYP1A2; CYP1B1; 7-ethoxyresorufin; 7-ethoxyresorufin *O*-dealkylation; 7-methoxyresorufin, 7-benzyloxyresorufin; 7-pentoxyresorufin.

## 1. Introduction

The human CYP1 family consists of three enzymes: CYP1A1, CYP1A2, and CYP1B1 ***(1)***. CYP1A1 is absent or present at very low levels in human liver ***(2,3)***, but its expression is readily detectable in lung ***(4,5)***. By contrast, CYP1A2 is expressed constitutively in human liver ***(2,3)*** and is absent in lung ***(4,5)***. CYP1B1 protein is undetectable in human liver ***(6)***, but CYP1B1 mRNA is expressed in liver ***(6)***, although the levels are greater in extrahepatic tissues such as kidney, prostate, and breast ***(7)***. Exposure to polycyclic aromatic hydrocarbons such as those found in cigarette smoke induces the expression of both CYP1A1 and CYP1A2 ***(3,8)***. CYP1B1 is also inducible by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, as demonstrated by cell culture experiments

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***(9,10)***. The chemical inhibitor α-naphthoflavone, which is a potent inhibitor of CYP1A1 and CYP1A2 ***(11)***, also inhibits CYP1B1 and with similar efficacy and potency ***(12)***. cDNA-expressed CYP1A1, CYP1A2, and CYP1B1 are each active in the oxidation of theophylline ***(12)***, caffeine ***(12,13)***, estradiol ***(14,15)***, benzo[*a*]pyrene ***(12)***, and 7-ethoxyresorufin ***(12)***. With 7-ethoxyresorufin as substrate, the rank order of specific activity (pmoles/[minute·nmoles] of cytochrome P450 [P450]) is CYP1A1 > CYP1B1 > CYP1A2 ***(12)***. 7-Ethoxyresorufin *O*-deethylation activity can be a useful and convenient catalytic monitor when conducting a comparative study with a panel of these three recombinant CYP1 enzymes ***(16)*** (*see* **Note 1**). Experiments with CYP1A-selective inhibitory antibodies indicate that CYP1A2 is a major contributor to 7-ethoxyresorufin *O*-deethylation activity in human liver microsomes ***(17,18)***. Accordingly, this activity is frequently used as a marker for human hepatic CYP1A2 (*see* **Note 2**). This chapter describes a 7-ethoxyresorufin *O*-deethylation assay (*see* **Note 3**), which is based on the spectrofluorometric method of Burke and Mayer ***(19)***. This reaction can also be measured by high-performance liquid chromatography with fluorescence detection ***(18)***.

## 2. Materials

1. Substrate: 7-ethoxyresorufin (mol wt = 241.2) (Molecular Probes, Eugene, OR). Prepare a 1 m*M* (0.24 mg/mL) stock solution dissolved in dimethyl sulfoxide (DMSO) (*see* **Notes 4** and **5**).
2. Metabolite standard: resorufin (sodium salt; mol wt = 235.2) (Molecular Probes).
3. Assay buffer: 100 m*M* potassium phosphate, pH 7.4, containing 1.5 m*M* EDTA.
4. Cofactor: 50 m*M* (42 mg/mL) nicotinamide adenine dinucleotide phosphate (NADPH) stock solution. Prepare fresh and store on ice (*see* **Notes 6–8**).
5. Enzymes: for example, cDNA-expressed CYP1A1, CYP1A2, and CYP1B1 (BD Biosciences, Discovery Labware, Woburn, MA) or human liver microsomes can be used. Dilute in assay buffer to a working concentration of 1 mg/mL and place on ice (*see* **Note 9**).
6. Spectrofluorometer connected to a circulating water bath.

## 3. Methods

1. Add the following to a cuvet (*see* **Note 10**) placed in a spectrofluorometer connected to a circulating water bath set at 37°C: 1.97 mL of assay buffer, 10 µL of 0.5 m*M* 7-ethoxyresorufin (0.25 µ*M* final concentration; *see* **Note 11**), 10 µL of diluted enzymes (10 µg of protein; *see* **Notes 12** and **13**).
2. Record the background fluorescence reading of the sample at an excitation wavelength of 530 nm and an emission wavelength of 582 nm (*see* **Note 14**).
3. Add 10 µL of 50 m*M* NADPH (0.25 m*M* final concentration) to the cuvet and mix to initiate the enzymatic reaction.
4. Record the progress of the enzymatic reaction for 2–3 min (*see* **Note 15**). Calculate the reaction rate (fluorescence units of resorufin product formed per unit of time) from the linear portion of the progress curve.
5. Calculate the net fluorescence of each unknown sample by subtracting the background fluorescence reading from the reading for each unknown sample.
6. Prepare standards by adding 10 µL of a known amount (e.g., 0, 0.1, 0.25, 0.5, 1, and 2 nmol) of authentic resorufin metabolite (dissolved in DMSO) to a separate cuvet con-

taining the complete incubation mixture but with heat-inactivated enzymes. Record the fluorescence reading.

7. Calculate the net fluorescence of each standard sample by subtracting the background fluorescence reading from the reading for each standard sample.
8. Plot a standard curve of net fluorescence vs nanomoles of authentic resorufin metabolite. Calculate the slope and intercept by linear regression analysis.
9. Use the standard curve to calculate 7-ethoxyresorufin *O*-deethylation activity for each unknown sample. Express the results as nanomoles of product formed/(minute·milligrams of protein) or as nanomoles of product formed/(minute·nanomoles of total P450).

## 4. Notes

1. 7-Ethoxycoumarin *O*-deethylation can also be used as a measure of the catalytic activity of each of the three recombinant CYP1 enzymes, CYP1A1, CYP1A2, and CYP1B1. However, CYP1A1, CYP1A2, and CYP1B1 are, respectively, approximately threefold, twofold, and sixfold more active in 7-ethoxyresorufin *O*-deethylation than in 7-ethoxycoumarin *O*-deethylation ***(12)***.
2. The apparent $K_m$ values for 7-ethoxyresorufin *O*-deethylation catalyzed by cDNA-expressed human CYP1A1 and CYP1A2 are 0.09 and 0.076 μ*M*, respectively ***(16)***. However, CYP1A1 is likely to account for the majority of 7-ethoxyresorufin *O*-deethylation activity in human lung microsomes because it is CYP1A1, rather than CYP1A2, that is expressed in human lung ***(4,5)***. By contrast, CYP1A2 is likely the primary 7-ethoxyresorufin *O*-deethylase in human liver microsomes because CYP1A2 is expressed in human liver ***(2,3,20)***, whereas CYP1A1 is either absent or present at very low levels in this tissue ***(2,20)***. CYP1B1 is not expected to contribute to hepatic microsomal 7-ethoxyresorufin *O*-dealkyation activity because this protein is not detectable in human liver ***(6)***.
3. The methods described using 7-ethoxyresorufin as substrate can be extended to three other alkoxyresorufins—7-methoxyresorufin, 7-benzyloxyresorufin, and 7-pentoxyresorufin—whose P450-catalyzed *O*-dealkylation activities can be assayed using essentially the same method described in **Subheading 3.** All four alkoxyresorufins yield the same fluorescent metabolite, resorufin. CYP1A2 appears to account for the majority of human hepatic microsomal 7-methoxyresorufin *O*-dealkylation activity, as judged by immunoinhibition experiments with heterologous anti-CYP1A2 antibodies ***(18)***. However, recombinant human CYP1A1 and CYP1A2 both metabolize 7-methoxyresorufin and with similar apparent $K_m$ (0.2–0.4 μ*M*) and similar apparent $V_{max}$ (approx 0.5 nmol/[min·mg of microsomal protein]) values ***(13)***. CYP3A, CYP1A, and CYP2A can all contribute to human hepatic microsomal 7-benzyloxyresorufin *O*-dealkylation activity ***(18)***, which is consistent with the observation that multiple recombinant human P450 enzymes are active catalysts in the oxidation of 7-benzyloxyresorufin ***(21,22)***. 7-Pentoxyresorufin *O*-dealkylation activity is very low but detectable in human liver microsomes (approx 3% the rate of 7-ethoxyresorufin *O*-dealkylase activity) ***(23)***, but it is not known which human hepatic P450 enzyme(s) is responsible for this activity.
4. 7-Ethoxyresorufin and resorufin are light sensitive. Store these chemicals in the dark.
5. Resorufin and alkoxyresorufin stock solutions prepared in DMSO can be stored in the dark and at room temperature for up to 1 yr without degradation ***(19)***.
6. Prepare fresh solutions of NADPH for each experiment. NADPH is light- and pH sensitive.
7. Alkoxyresorufin *O*-dealkylation assays can also be carried out with an NADPH-generating system (e.g., $NADP^+$, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) in place of NADPH.

8. Artificially high 7-ethoxyresorufin *O*-deethylation activity is obtained with assay protocols that use acid to precipitate microsomal protein because NADPH reacts with 7-ethoxyresorufin in the presence of acid to form a product that fluoresces at the same wavelength as resorufin ***(24)***.
9. Prepare only sufficient amounts of dilute human liver microsomes for each experiment. The remainder of the undiluted microsomes can be stored at –80°C for future use ***(25)***.
10. 7-Ethoxyresorufin *O*-deethylation activity in cultured cells (e.g., hepatocytes) can be measured directly in culture plates by using a fluorescence multiwell plate reader ***(26,27)***.
11. Optimal 7-ethoxyresorufin concentration is ≤2.5 µ*M*. Reduced activity is observed at a substrate concentration >2.5 µ*M*, owing to inhibition of 7-ethoxyresorufin *O*-deethylation activity by the enzymatic product, resorufin ***(24)***.
12. With some P450 protein expression systems, it is necessary to reconstitute the recombinant P450 protein with NADPH–cytochrome P450 reductase, cytochrome-$b_5$, and lipid prior to initiating substrate oxidation ***(12,28,29)***. Conduct preliminary experiments to establish the amount of each of these required for optimal catalytic activity.
13. Conduct preliminary experiments to ensure that the assay is linear with respect to microsomal protein concentration.
14. Determine the optimal excitation wavelength and emission wavelength to be used with each particular spectrofluorometer by examining the excitation and emission spectra ***(30)*** of 7-ethoxyresorufin and resorufin generated by that instrument.
15. 7-Alkoxyresorufin *O*-dealkylation assays can also be carried out using a modified protocol, in which methanol is used to stop the enzymatic reaction after a fixed incubation time, and the precipitated protein is centrifuged and the fluorescence of the supernatant is then measured ***(22,24)***.

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# 9

## Spectrofluorometric Analysis of CYP2A6-Catalyzed Coumarin 7-Hydroxylation

**David J. Waxman and Thomas K. H. Chang**

**Summary**

A spectrofluorometric method is described for the determination of CYP2A6-catalyzed coumarin 7-hydroxylation. Following acidification of the reaction mixture, the enzymatic product, 7-hydroxycoumarin, is recovered by a double-extraction procedure and assayed using an excitation wavelength of 370 nm and an emission wavelength of 450 nm. This assay is applicable to enzymatic studies of cDNA-expressed CYP2A6 and can be used to monitor coumarin 7-hydroxylation activity in microsomes prepared from liver and other tissues and in isolated hepatocytes and cultured cells that express this cytochrome P450 activity.

**Key Words:** Cytochrome P450; CYP2A6; coumarin; coumarin 7-hydroxylation; 7-hydroxycoumarin.

## 1. Introduction

CYP2A6 has been isolated and purified to apparent homogeneity from human liver ***(1,2)***. The level of CYP2A6 protein expressed in liver is low (~4% of total hepatic cytochrome P450 [P450] content) ***(3)***, with substantial interindividual variation in enzyme levels ***(3–7)***. CYP2A6 is primarily expressed in liver; it is apparently absent from several extrahepatic tissues, including adult human lung, colon, breast, kidney, and placenta microsomes. In primary cultures of human hepatocytes, CYP2A6 is inducible by diverse drugs, including phenobarbital, dexamethasone, and rifampin ***(8)***. Substrates for CYP2A6 include nicotine ***(9)***, chloroform ***(10)***, and coumarin ***(2)***. In fact, experiments with individual cDNA-expressed human P450 forms have indicated that CYP2A6 is a major catalyst of coumarin 7-hydroxylation ***(11)***. Among the other recombinant human P450s examined, CYP1A1, 1A2, 2C8, 2C9, 2D6, 2E1, 3A4, and 3A5 are catalytically inactive with respect to coumarin 7-hydroxylation, whereas CYP2B6 is active at only approx 5% the rate of CYP2A6 ***(11)***. Immunoinhibition studies have validated the use of coumarin 7-hydroxylase activity as a diagnostic catalytic marker for human hepatic CYP2A6. Anti-human CYP2A6 polyclonal IgG ***(2)***

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and monoclonal antibody to CYP2A6 *(12)* inhibit coumarin 7-hydroxylase activity by >90% in human liver microsomes, indicating that CYP2A6 is the principal and perhaps the sole catalyst of coumarin 7-hydroxylation activity in human liver. This activity can also be inhibited by a variety of compounds, including tranylcypromine *(13)*, α-naphthoflavone *(13–16)*, and diethyldithiocarbamate *(13,15,16)*, but 8-methoxypsoralen is a CYP2A6-selective mechanism-based inactivator *(17)*. This chapter describes a modification *(18)* of a spectrofluorometric method *(19)* for the determination of coumarin 7-hydroxylation. The product of this reaction, 7-hydroxycoumarin, can also be quantified by high-performance liquid chromatography (HPLC) with fluorometric detection *(20)* or HPLC-tandem mass spectrometry *(21)*.

## 2. Materials

1. Assay buffer: 100 m*M* potassium phosphate, pH 7.4, containing 0.1 m*M* EDTA (*see* **Note 1**).
2. Substrate: coumarin (1,2-benzopyrone; mol wt = 146.1) (Sigma-Aldrich, St. Louis, MO). Prepare a 1 m*M* (0.15 mg/mL) stock solution dissolved in assay buffer.
3. Metabolite standard: 7-hydroxycoumarin (umbelliferone; mol wt = 162.1) (Sigma-Aldrich).
4. Cofactor: 10 m*M* (8.3 mg/mL) nicotinamide adenine dinucleotide phosphate (NADPH) stock solution. Prepare fresh and keep on ice (*see* **Notes 2–4**).
5. Enzymes: cDNA-expressed CYP2A6 (BD Biosciences, Discovery Labware, Woburn, MA) or human liver microsomes. Dilute in assay buffer to a working concentration of 1 mg of protein/mL and keep on ice (*see* **Note 5**).
6. Deproteinizing agent: 2 *M* HCl.
7. Extraction solvent: chloroform.
8. Back-extraction solution: 30 m*M* (6 mg/mL) sodium borate ($Na_2B_4O_7$), pH 9.2.

## 3. Methods

1. Add the following to each incubation tube (total incubation volume of 200 µL) (*see* **Note 6**): 150 µL of assay buffer, 10 µL of 1 m*M* coumarin (50 µ*M* final concentration) (*see* **Notes 7** and **8**), 20 µL of diluted enzymes (to give 20 µg of protein) (*see* **Notes 9** and **10**).
2. Prewarm incubation tubes to 37°C, and add 20 µL of 10 m*M* NADPH (1 m*M* final concentration) to initiate enzymatic reaction (stagger each incubation with 15-s delay intervals).
3. Incubate samples at 37°C for 15–30 min in a shaking water bath (*see* **Note 11**).
4. Add 25 µL of ice-cold 2 *M* HCl to stop the enzymatic reaction and place the incubation tube on ice.
5. Extract with 450 µL of chloroform while vortexing for 30 s (*see* **Note 12**).
6. Centrifuge the extraction tubes at 3000*g* for 5 min.
7. Transfer 300 µL of the organic phase (bottom layer) to a clean test tube and back-extract with 1 mL of 30 m*M* sodium borate (pH 9.2).
8. Repeat **step 6**.
9. Remove the top layer and measure the fluorescence at an excitation wavelength of 370 nm and an emission wavelength of 450 nm (*see* **Note 13**).
10. Prepare blank incubation tubes by adding the complete incubation mixture but with heat-inactivated enzymes. Process the blank incubation tubes per **steps 3–9**.
11. Prepare standards by adding a known amount (e.g., 0, 0.1, 0.2, 0.4, 0.8, and 1.2 nmol) of authentic 7-hydroxycoumarin metabolite to tubes containing complete incubation mix-

ture but with heat-inactivated enzymes. Process the incubation tubes containing the standards per **steps 3–9**.

12. Calculate the net fluorescence of each unknown sample and standard by subtracting the fluorescence reading of the blank from that of the unknown or standard.
13. Plot a standard curve of net fluorescence vs the amount of authentic 7-hydroxycoumarin (e.g., 0, 0.1, 0.2, 0.4, 0.8, and 1.2 nmol), and determine the amount of product formation in each unknown sample by linear regression analysis.
14. Calculate coumarin 7-hydroxylation activity and express it as nanomoles of product formed/(minute·milligrams of microsomal protein) or as nanomoles of product formed/(minute·nanomoles of total P450) (*see* **Notes 14** and **15**).

## 4. Notes

1. Coumarin 7-hydroxylation activity is highly dependent on the ionic strength of the buffer system ***(4,22)***.
2. Prepare a fresh solution of NADPH for each experiment. NADPH is light- and pH-sensitive.
3. Coumarin 7-hydroxylation assays can also be carried out using an NADPH-generating system (e.g., $NADP^+$, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase ***[4]*** or $NADP^+$, isocitric acid, and isocitric acid dehydrogenase ***[23]***) in place of NADPH.
4. CYP2A6 is substantially less active (30–50% lower activity) with some lots of $NADP^+$ (cat. no. N0505; Sigma-Aldrich) and with the monosodium salt of D-glucose 6-phosphate (cat. no. G7879; Sigma-Aldrich). A similar inhibitory effect has been observed for rat CYP2A1-catalyzed testosterone 7α-hydroxylase activity (unpublished observation).
5. Dilute only sufficient amounts of microsomes for each experiment. The remainder of the undiluted microsomes can be stored at –80°C for future use ***(24)***.
6. The formation of 7-hydroxycoumarin can be assayed directly in cultured CYP2A6-expressing cells ***(25)*** and primary cultures of human hepatocytes ***(26)*** using a fluorescence multiwell plate reader after incubating the cells with coumarin in a multiwell plate at 37°C.
7. According to results from immunoinhibition experiments, coumarin 7-hydroxylase activity is selective for human liver microsomal CYP2A6 regardless of whether the assay is performed at a substrate concentration of 0.02 ***(2)***, 0.05 ***(4)***, 0.6 ***(23)***, or 1 m*M* ***(27)***.
8. The apparent $K_m$ value for coumarin 7-hydroxylation catalyzed by cDNA-expressed CYP2A6 or by human liver microsomes is approx 0.5 μ*M* ***(13)***. Therefore, the suggested substrate concentration (50 μ*M*) to be used in this assay is approx 100-fold greater than the apparent $K_m$.
9. Conduct preliminary experiments to ensure that the assay is linear with respect to microsomal protein concentration.
10. With some P450 protein expression systems, it is necessary to reconstitute the recombinant P450 protein with NADPH–cytochrome P450 reductase, cytochrome-$b_5$, and lipid prior to initiating substrate oxidation ***(22)***. Conduct preliminary experiments to establish the amount of each of these required for optimal catalytic activity.
11. Conduct preliminary experiments to ensure that the assay is linear with respect to incubation time.
12. "Wet" pipet tips with organic solvent prior to use.
13. Determine the optimal excitation wavelength and emission wavelength to be used with each particular spectrofluorometer by examining the excitation and emission spectra of coumarin and 7-hydroxycoumarin ***(28)*** generated by that instrument.

14. Typical coumarin 7-hydroxylase activity levels found in human liver microsomes range from <0.01 to 4.75 nmol/(min·mg) of microsomal protein *(29)*.
15. Methanol at a final concentration of 1–5% (v/v) does not inhibit human liver microsomal coumarin 7-hydroxylase activity when the assay is conducted at a substrate concentration of 50 μ*M* *(13)*. However, at a lower substrate concentration (0.5 μ*M*), methanol at a final concentration of >1% (v/v) decreases this activity. By comparison, inclusion of a 1% (v/v) final concentration of dimethyl sulfoxide (DMSO), dimethylformamide, 2-propanol, ethanol, acetonitrile, acetone, tetrahydrofuran, or dioxane in the incubation mixture results in inhibition of coumarin 7-hydroxylase activity in human liver microsomes, especially at substrate concentration of 0.5 μ*M*. No appreciable inhibition occurs with DMSO, ethanol, or acetonitrile at a final concentration of ≤0.5% (v/v) *(30,31)*.

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# 10

# Determination of CYP2B6 Component of 7-Ethoxy-4-Trifluoromethylcoumarin *O*-Deethylation Activity in Human Liver Microsomes

**Thomas K. H. Chang, Charles L. Crespi, and David J. Waxman**

**Summary**

The cytochrome P450 enzyme CYP2B6 plays an important role in the metabolism of structurally diverse drugs, including the anticancer drug cyclophosphamide, and may be an important determinant of clinical responses to these agents. A spectrofluorometric method is described for the determination of CYP2B6-catalyzed 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylation activity in human liver microsomes. The specificity of this method for CYP2B6 is increased by the use of inhibitory antibodies to CYP1A2, CYP2C, and CYP2E1, which block the contributions of these higher-$K_m$ enzymes to human liver microsomal metabolism of 7-ethoxy-4-trifluoromethylcoumarin. The enzymatic product, 7-hydroxy-4-trifluoromethylcoumarin, is monitored by fluorescence using an excitation wavelength of 410 nm and an emission wavelength of 510 nm. This approach can be modified to assay the catalytic activity of cDNA-expressed CYP2B6.

**Key Words:** Cytochrome P450; CYP2B6; 7-ethoxy-4-trifluoromethylcoumarin; 7-hydroxy-4-trifluoromethylcoumarin; 7-ethoxy-4-trifluoromethylcoumarin; *O*-deethylation.

## 1. Introduction

CYP2B6 protein has been isolated and purified from human liver *(1,2)*. This cytochrome P450 (P450) accounts for <1% of total P450 content in human liver in a panel of 60 individual human liver microsome samples *(2)*. However, large interindividual variability has been reported for hepatic CYP2B6 mRNA *(3)* and protein *(1,2,4)*. Experiments with primary cultures of human hepatocytes have shown that CYP2B6 is inducible by treatment of the cells with phenobarbital *(5,6)*, dexamethasone *(5,7)*, or rifampin *(5–7)*. By contrast, the level of this P450 in hepatocyte cultures does not appear to be altered by other P450 inducers such as β-naphthoflavone *(5)*, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin *(5)*, or pregnenolone 16α-carbonitrile *(7)*. Several xenobiotic substrates have been identified for cDNA-expressed CYP2B6, including

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7-ethoxycoumarin ***(3,8,9)***, benzo[*a*]pyrene ***(10)***, phenanthrene ***(11)***, and methoxychlor ***(12)***. Drugs that are metabolized by CYP2B6 include cyclophosphamide ***(9)***, propofol ***(13,14)***, bupropion ***(15)***, and lidocaine ***(16)***. (*S*)-mephenytoin *N*-demethylation is used as a catalytic monitor for CYP2B6 in human liver microsomes ***(17)***, but the enzyme assay must be conducted at a substrate concentration >1000 μ*M* in order to minimize the contribution of the lower-$K_m$ enzyme CYP2C9 ***(18)***. Bupropion hydroxylation is also selective for CYP2B6 in human liver microsomes ***(19,20)***, as is 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylation ***(4)***. Although several P450 enzymes (i.e., CYP1A, CYP2B6, CYP2C, and CYP2E1) contribute to 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylation in human liver microsomes ***(4,21,22)***, this activity can be assayed under conditions in which it is CYP2B6 selective, i.e., by using a low substrate concentration (5 μ*M*) and by preincubating human liver microsomes with a mixture of inhibitory antibodies to block the activities of CYP1A, CYP2C, and CYP2E1 enzymes ***(4)***.

This chapter describes a spectrofluorometric method for determination of the CYP2B6 component of human liver microsomal 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylation activity. Simple modification of this assay method can be introduced to assay cDNA-expressed CYP2B6-catalyzed 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylation ***(4)***. Because of the simplicity of a fluorescence-based assay, this reaction is often used in CYP2B6 inhibition studies with the cDNA-expressed enzyme (e.g., *see* **refs. *23*** and ***24***).

## 2. Materials

1. Assay buffer: 100 m*M* potassium phosphate, pH 7.4.
2. Substrate: 7-ethoxy-4-trifluoromethylcoumarin (mol wt = 258.2) (Enzyme Systems Products, Livermore, CA) (*see* **Note 1**). Prepare a 25 m*M* (6.4 mg/mL) stock solution dissolved in methanol (*see* **Notes 2** and **3**). Dilute to 50 μ*M* with assay buffer.
3. Metabolite standard: 7-hydroxy-4-trifluoromethylcoumarin (mol wt = 230.2) (Enzyme Systems Products) (*see* **Note 1**).
4. Cofactor-generating system: 26 m*M* (20 mg/mL) $NADP^+$ (*see* **Note 4**), 66 m*M* (20 mg/mL) D-glucose-6-phosphate, and 66 m*M* (13.3 mg/mL) magnesium chloride ($MgCl_2 \cdot 6H_2O$); glucose-6-phosphate dehydrogenase (40 U/mL) in 5 m*M* (1.5 mg/mL) sodium citrate monohydrate.
5. cDNA-expressed CYP2B6 (BD Biosciences, Discovery Labware, Woburn, MA). Enzymes: dilute human liver microsomes in assay buffer to a working concentration of 1 mg of microsomal protein/mL and keep on ice (*see* **Note 5**).
6. Antibodies:
    a. Anti-CYP1A2 (polyclonal antibody developed in rabbit [Daiichi, Tokyo, Japan] and available from BD Biosciences, Discovery Labware).
    b. Anti-CYP2C (polyclonal antibody developed in goat [Daiichi] and available from BD Biosciences, Discovery Labware).
    c. Anti-CYP2E1 (monoclonal antibody developed in mouse, available from BD Biosciences, Discovery Labware).
7. Deproteinizing agent: 20% (v/v) trichloroacetic acid (TCA).
8. 100 m*M* Tris-HCl, pH 9.0.
9. Spectrofluorometer.
10. Water bath.

## 3. Methods

1. Mix 10 µL of the diluted human liver microsomes with 4 µL of the antibody mixture (*see* **Subheading 2., item 6**). Incubate for 30 min on ice.
2. Add the following to each incubation tube (total incubation volume of 200 µL):
   a. 154 µL of assay buffer.
   b. 20 µL of 50 µ*M* 7-ethoxy-4-trifluoromethylcoumarin (5 µ*M* final concentration, *see* **Note 6**).
   c. 10 µL of a solution containing 26 m*M* $NADP^+$, 66 m*M* D-glucose-6-phosphate, and 66 m*M* magnesium chloride.
   d. 2 µL of glucose-6-phosphate dehydrogenase (40 U/mL) in 5 m*M* sodium citrate.
3. Prewarm incubation tubes to 37°C, and add 14 µL of the mixture containing the human liver microsomes and the inhibitory antibodies to inititate the enzymatic reaction (stagger each incubation with 15-s delay intervals) (*see* **Note 7**).
4. Incubate the samples at 37°C for 20–30 min in a water bath (*see* **Note 8**).
5. Add 40 µL of 20% (v/v) TCA to stop the enzymatic reaction, and place the incubation tube on ice (*see* **Note 9**).
6. Centrifuge the reaction mixture at 10,000*g* for 3 min.
7. Transfer 100 µL of the supernatant to a clean test tube containing 1.9 mL of 100 m*M* Tris-HCl, pH 9.0, and vortex.
8. Measure the fluorescence at an excitation wavelength of 410 nm and an emission wavelength of 510 nm (*see* **Note 10**).
9. Prepare blank incubation tubes by adding the complete incubation mixture but with heat-inactivated microsomes. Process the blank incubation tubes per **steps 4–8**.
10. Prepare standards by adding a known amount (e.g., 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1 nmol) of authentic 7-hydroxy-4-trifluoromethylcoumarin metabolite to tubes containing the complete incubation mixture but with heat-inactivated microsomes. Process the incubation tubes containing the standards per **steps 4–8**.
11. Calculate the net fluorescence of each unknown sample and standard by subtracting the fluorescence reading of the blank from that of the unknown or standard.
12. Plot a standard curve of net fluorescence against the amount of authentic 7-hydroxy-4-trifluoromethylcoumarin and determine the amount of product formation in each unknown sample by linear regression analysis.
13. Calculate 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylation activity and express it as nanomoles of product formed/(minute·milligrams of microsomal protein) or as nanomoles of product formed/(minute·nanomoles of total P450) (*see* **Note 11**).

## 4. Notes

1. 7-Ethoxy-4-trifluoromethylcoumarin and 7-hydroxy-4-trifluoromethylcoumarin are light sensitive.
2. The limit of solubility of 7-ethoxy-4-trifluoromethylcoumarin in potassium phosphate buffer is approx 50 µ*M*, but this can be increased to approx 100 µ*M* by using dimethyl sulfoxide (DMSO) (0.2% [v/v] final concentration) as a vehicle. However, this concentration of DMSO results in an approx 5% decrease in enzyme activity ***(25)***. Ethanol is highly inhibitory to CYP2B6, with approx 50% inhibition observed at a final concentration of 0.3% ***(26)***. By comparison, methanol and acetonitrile at a final concentration of 0.3% (v/v) inhibits CYP2B6-catalyzed 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylation by 5 and 13%, respectively ***(26)***.

3. Stock solutions of 7-ethoxy-4-trifluoromethylcoumarin are stable for at least 1 yr when stored at –20°C ***(25)***.
4. 7-Ethoxy-4-trifluoromethylcoumarin *O*-deethylation assays can also be carried out with nicotinamide adenine dinucletide phosphate (NADPH) (e.g., 1 m*M* final concentration) instead of an NADPH-generating system (e.g., $NADP^+$, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase).
5. Dilute only sufficient amounts of microsomes for each experiment. The remainder of the undiluted microsomes can be stored at –80°C for future use ***(27)***.
6. The apparent $K_m$ value for cDNA-expressed CYP2B6-catalyzed 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylation is approx 3 μ*M* ***(4)***. The assay is conducted at a substrate concentration of 5 μ*M* in order to facilitate the efficacy of the antibodies against the higher-$K_m$ forms such as CYP2C19 (apparent $K_m$ = 17 μ*M*), CYP2C9 (apparent $K_m$ = 35 μ*M*), CYP2E1 (apparent $K_m$ = 6 μ*M*), and CYP2A6 (apparent $K_m$ > 50 μ*M*).
7. Conduct preliminary experiments to ensure that the assay is linear with respect to microsomal protein concentration.
8. Conduct preliminary experiments to ensure that the assay is linear with respect to incubation time.
9. The 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylation assay can also be performed using a direct continuous spectrofluorometric method ***(21,25)***.
10. Determine the optimal excitation wavelength and emission wavelength to be used with each particular spectrofluorometer by examining the excitation and emission spectra ***(25)*** of 7-ethoxy-4-trifluoromethylcoumarin and 7-hydroxy-4-trifluoromethylcoumarin generated by that instrument. If wide-bandwidth filters are used, there may be significant background fluorescence from NADPH.
11. The CYP2B6 component of human liver microsomal 7-ethoxy-4-trifluoromethylcoumarin *O*-deethylation activity at a substrate concentration of 5 μ*M* in a panel of 17 individual human liver microsome samples ranged from 0.02 to 0.5 nmol/(min·mg) of microsomal protein ***(4)***.

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# 11

## High-Performance Liquid Chromatography Analysis of CYP2C8-Catalyzed Paclitaxel 6α-Hydroxylation

**Charles L. Crespi, Thomas K. H. Chang, and David J. Waxman**

**Summary**

Paclitaxel (Taxol) is a naturally occurring member of the taxane family of antitumor drugs, which act by stabilizing microtubules. Paclitaxel is inactivated in human liver by a cytochrome P450 (P450)-catalyzed 6α-hydroxylation reaction. A reverse-phase, high-performance liquid chromatographic assay is described for the analysis of paclitaxel 6α-hydroxylation catalyzed by human liver microsomes or cDNA-expressed P450 enzyme CYP2C8. Analytical separations are achieved using a $C_{18}$ column with a linear gradient of 10–100% methanol, with detection at 230 nm. This method is applicable to enzymatic studies for determination of CYP2C8-catalyzed paclitaxel 6α-hydroxylation activity.

**Key Words:** Cytochrome P450; CYP2C8; paclitaxel; 6α-hydroxypaclitaxel; paclitaxel 6α-hydroxylation.

## 1. Introduction

CYP2C8 is a major CYP2C protein expressed in human liver ***(1–4)***. Considerable interindividual differences (approx 20-fold) have been observed in hepatic CYP2C8 protein content ***(5)***, and a bimodal distribution in CYP2C8 protein levels in a panel of human liver microsomes has been reported ***(6)***. CYP2C8 is subject to induction by phenobarbital, dexamethasone, and rifampin, as shown by experiments with primary cultures of human hepatocytes ***(4)***. Trimethoprim appears to be an enzyme-selective inhibitor of CYP2C8 ***(7)***. Substrates for this cytochrome P450 (P450) include retinol ***(6)***, retinoic acid ***(6,8)***, arachidonic acid ***(9,10)***, amiodarone ***(11)***, carbamazepine ***(12)***, morphine ***(13)***, methadone ***(14)***, amodiaquine ***(15)***, and paclitaxel ***(16–18)***. Oxidation of the anticancer drug paclitaxel to 6α-hydroxypaclitaxel is catalyzed by CYP2C8 but not by CYP 1A2, 2A6, 2B6, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, or 3A5 ***(16–18)***. Furthermore, CYP2C8 accounts for most of the paclitaxel 6α-hydroxylation activity in human liver microsomes, as determined by immunoinhibition experiments with

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monoclonal antibody to CYP2C8 *(19)*. Consequently, paclitaxel 6α-hydroxylation activity is used as a catalytic marker for human hepatic CYP2C8. This chapter describes a high-performance liquid chromatography (HPLC) assay for determination of paclitaxel 6α-hydroxylation activity.

## 2. Materials

### 2.1. Assay

1. Substrate: paclitaxel (mol wt = 853.9) (*see* **Notes 1** and **2**) (Calbiochem, La Jolla, CA). Prepare a 5 m*M* (4.3 mg/mL) stock solution dissolved in ethanol.
2. Metabolite standard: 6α-hydroxypaclitaxel (mol wt = 869.9) (*see* **Note 3**) (BD Biosciences, Discovery Labware, Woburn, MA).
3. Assay buffer: 100 m*M* potassium phosphate, pH 7.4.
4. Cofactor: 26 m*M* (20 mg/mL) $NADP^+$ (*see* **Note 4**), 66 m*M* (20 mg/mL) D-glucose-6-phosphate, and 66 m*M* (13.3 mg/mL) magnesium chloride ($MgCl_2 \cdot 6H_2O$); glucose-6-phosphate dehydrogenase (40 U/mL) in 5 m*M* (1.5 mg/mL) sodium citrate ($C_6H_5O_7Na_3 \cdot 2H_2O$).
5. Enzymes: for example, cDNA-expressed CYP2C8 (BD Biosciences, Discovery Labware) or human liver microsomes can be used. Dilute in assay buffer to a working concentration of 5 mg of protein/mL and keep on ice (*see* **Note 5**).
6. Deproteinizing agent: 100% acetonitrile.

### 2.2. High-Performance Liquid Chromatography

1. Mobile phase A: 10% (v/v) methanol (*see* **Note 6**).
2. Mobile phase B: 100% methanol (*see* **Note 6**).
3. Nucleosil $C_{18}$ column, 4.6 × 250 mm, 5-μm particle size (Sigma-Aldrich, St. Louis, MO) (*see* **Note 7**).
4. Ultraviolet detector at 230 nm.

## 3. Methods

1. Add the following to each incubation tube (total incubation volume of 200 μL):
   a. 178 μL of assay buffer.
   b. 0.4 μL of 5 m*M* paclitaxel (10 μ*M* final concentration; *see* **Notes 8** and **9**).
   c. 10 μL of a solution containing 26 m*M* $NADP^+$, 66 m*M* D-glucose-6-phosphate, and 66 m*M* magnesium chloride.
   d. 2 μL of glucose-6-phosphate dehydrogenase (40 U/mL) in 5 m*M* sodium citrate.
2. Prewarm incubation tubes to 37°C, and add 10 μL of diluted enzymes (50 μg of protein) to initiate enzymatic reaction (stagger each incubation with 15-s delay intervals) (*see* **Notes 10** and **11**).
3. Incubate the samples at 37°C for 30 min in a water bath (*see* **Note 12**).
4. Add 50 μL of ice-cold 100% acetonitrile to stop the enzymatic reaction and place the incubation tubes on ice.
5. Centrifuge the reaction mixture at 12,000*g* for 4 min.
6. Inject 50–150 μL of supernatant onto the HPLC column.
7. Run the column at 45°C (*see* **Note 13**) at a flow rate of 1 mL/min with a linear gradient of 45% mobile phase A, 55% mobile phase B to 35% phase A, 65% phase B over 20 min, then for a further 5 min with 35% phase A, 65% phase B. Under these conditions, the retention times are 22 min for 6α-hydroxypaclitaxel and 25.5 min for paclitaxel.

8. Prepare blank incubation tubes by adding the complete incubation mixture but with heat-inactivated enzymes. Process the blank incubation tubes per **steps 3–7**.
9. Prepare standards by adding a known amount (e.g., 0.1, 0.2, 0.5, and 1 nmol) of authentic 6α-hydroxypaclitaxel metabolite to tubes containing the complete incubation mixture but with heat-inactivated enzymes (*see* **Note 14**). Process the incubation tubes containing the standards per **steps 3–7**.
10. Plot a standard curve of the peak area (or peak height) of metabolite standard vs the amount of authentic standard added. Determine the amount of product formation in each unknown sample by linear regression analysis.
11. Calculate paclitaxel 6α-hydroxylation activity and express it as nanomoles of product formed/(minute·milligrams of microsomal protein) or as nanomoles of product formed/(minute·nanomoles of total P450).
12. Calculate net enzyme activity by subtracting the activity in the blank from each of the unknown samples (*see* **Notes 15** and **16**).

## 4. Notes

1. Store paclitaxel at 4°C. Paclitaxel is light sensitive.
2. Paclitaxel solution may be stored up to 1 yr at –20°C. A significant amount of the drug adsorbs to glass, polypropylene, and polystyrene containers ***(20)***.
3. Storage of solid and solutions of 6α-hydroxypaclitaxel metabolite standard at ≤–20°C is recommended. A methanol solution of 6α-hydroxypaclitaxel stored at room temperature shows approx 30% degradation after 14 d. No degradation of an acetonitrile solution or the dry solid is observed under the same room temperature condition.
4. Paclitaxel 6α-hydroxylation assays can also be carried out with nicotinamide adenine dinucleotide phosphate (NADPH) (e.g., 1 m*M* final concentration) instead of an NADPH-generating system (e.g., $NADP^+$, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase).
5. Prepare only sufficient amounts of dilute microsomes for each experiment. The remainder of the undiluted microsomes can be stored at –80°C for future use ***(21)***.
6. Mobile phases containing acetonitrile may be used instead of methanol, but this requires adjustment of mobile-phase gradient conditions.
7. Paclitaxel and 6α-hydroxypaclitaxel can be separated by $C_{18}$ columns from other manufacturers, but this may require adjustment of mobile-phase gradient conditions. The other major metabolite, *p*-3'-hydroxypaclitaxel (BD Biosciences, Discovery Labware), should be tested to ensure that it does not comigrate with 6α-hydroxypaclitaxel.
8. Paclitaxel concentrations of >20 μ*M* result in decreased metabolite formation by human liver microsomes ***(17)***.
9. The apparent $K_m$ for paclitaxel 6α-hydroxylation by cDNA-expressed CYP2C8 and by human liver microsomes is approx 5 μ*M* ***(16)***.
10. With some P450 protein expression systems, it is necessary to reconstitute the recombinant P450 protein with NADPH–cytochrome P450 reductase, cytochrome-$b_5$, and lipid before initiating substrate oxidation ***(3)***. Conduct preliminary experiments to establish the amount of each of these required for optimal catalytic activity.
11. Conduct preliminary experiments to ensure that the assay is linear with respect to microsomal protein concentration.
12. Conduct preliminary experiments to ensure that the assay is linear with respect to incubation time.

13. Column temperature can range from room temperature to 50°C. The use of a controlled, elevated temperature provides greater reproducibility in retention times and lower column back-pressures.
14. The final concentration of 6α-hydroxypaclitaxel metabolite standard can be determined by absorbance at 230 nm using a molar extinction coefficient of 28.2 $cm^{-1}$ m$M^{-1}$. After determination of absorbance, dilute the standard to 50% methanol/50% water before use. Diluted standard can be stored at –20°C.
15. The median paclitaxel 6α-hydroxylase activity in a panel of 49 human liver microsomes was 1.09 nmol/(h·mg of protein) at a substrate concentration of 20 μ*M* ***(17)***.
16. Methanol, ethanol, dimethyl sulfoxide, and acetonitrile at ≤0.3% (v/v) do not appreciably inhibit CYP2C8-catalyzed paclitaxel 6α-hydroxylation ***(22)***.

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# 12

# Determination of CYP2C9-Catalyzed Diclofenac 4'-Hydroxylation by High-Performance Liquid Chromatography

**Charles L. Crespi, Thomas K. H. Chang, and David J. Waxman**

**Summary**

A reverse-phase, high-performance liquid chromatography method is described for quantification of diclofenac 4'-hydroxylation catalyzed by human liver microsomes or cDNA-expressed CYP2C9. Analytical separation is achieved using a $C_{18}$ column developed with a gradient of 30% acetonitrile and 2 m*M* perchloric acid in water to 100% methanol, with detection at 280 nm. This method is applicable to enzymatic studies for determination of CYP2C9-catalyzed diclofenac 4'-hydroxylation activity.

**Key Words:** Cytochrome P450; CYP2C9; diclofenac; 4'-hydroxydiclofenac; diclofenac 4'-hydroxylation.

## 1. Introduction

CYP2C9 is a major CYP2C enzyme expressed in human liver ***(1)***. Experiments with primary cultures of human hepatocytes have suggested that CYP2C9 protein expression can be increased in cells treated with a cytochrome P450 (P450) inducer such as phenobarbital, dexamethasone, or rifampin ***(2)***. This is consistent with the clinical finding that rifampin increases the total body clearance of tolbutamide ***(3)***, which is metabolized almost entirely by hepatic CYP2C9 ***(4–6)***. Other drugs that are metabolized by CYP2C9 include cyclophosphamide ***(7)***, ifosfamide ***(7)***, phenytoin ***(4,8)***, warfarin ***(9)***, diclofenac ***(10)***, celecoxib ***(11)***, and valproic acid ***(12,13)***. Sulfaphenazole is a CYP2C9-selective chemical inhibitor ***(14–17)*** and inhibition experiments with this compound have indicated that human liver microsomal diclofenac 4'-hydroxylation is selectively catalyzed by CYP2C9 ***(10)***. The selectivity of this metabolic reaction in human liver microsomes has been confirmed by immunoinhibition experiments with monoclonal antibodies to CYP2C9 ***(6)***.

This chapter describes a high-performance liquid chromatography (HPLC) assay for determination of diclofenac 4'-hydroxylation activity. Methods for other CYP2C9

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assays such as tolbutamide 4'-hydroxylation *(18)* and (*S*)-warfarin 7-hydroxylation *(19,20)* can be found in the cited references. These assays typically require higher microsomal protein concentrations.

## 2. Materials

### 2.1. Assay

1. Assay buffer: 100 m*M* Tris-HCl, pH 7.5.
2. Substrate: diclofenac (sodium salt; mol wt = 318.1) (Sigma-Aldrich, St. Louis, MO). Prepare a 0.4 m*M* (0.127 mg/mL) stock solution dissolved in assay buffer (*see* **Note 1**).
3. Metabolite standard: 4'-hydroxydiclofenac (BD Biosciences, Discovery Labware, Woburn, MA).
4. Cofactor-generating system: 26 m*M* (20 mg/mL) $NADP^+$ (*see* **Note 2**), 66 m*M* (20 mg/mL) D-glucose-6-phosphate, 66 m*M* (13.3 mg/mL) magnesium chloride ($MgCl_2 \cdot 6H_2O$); glucose-6-phosphate dehydrogenase (40 U/mL) in 5 m*M* (1.5 mg/mL) sodium citrate ($C_6H_5O_7Na_3 \cdot 2H_2O$).
5. Enzymes: for example, cDNA-expressed CYP2C9 (BD Biosciences, Discovery Labware) or human liver microsomes can be used. Dilute in assay buffer to a working concentration of 2.5 mg of protein/mL and keep on ice (*see* **Note 3**).
6. Deproteinizing agent: 94% acetonitrile/6% glacial acetic acid.

### 2.2. High-Performance Liquid Chromatography

1. Mobile phase A: 30% acetonitrile, 70% water, 2 m*M* perchloric acid (in water) (*see* **Note 4**).
2. Mobile phase B: 100% methanol.
3. Nucleosil $C_{18}$ column, 4.6 × 250 mm, 5-μm particle size (Sigma-Aldrich) (*see* **Note 5**).
4. Ultraviolet detector at 280 nm.

## 3. Methods

1. Add the following to each incubation tube (total incubation volume of 200 μL):
    a. 118 μL of assay buffer.
    b. 50 μL of 0.4 m*M* diclofenac (100 μ*M* final concentration, *see* **Note 6**).
    c. 10 μL of a solution containing 26 m*M* $NADP^+$, 66 m*M* D-glucose-6-phosphate, and 66 m*M* magnesium chloride.
    d. 2 μL of glucose-6-phosphate dehydrogenase (40 U/mL) in 5 m*M* sodium citrate.
2. Prewarm incubation tubes to 37°C and add 20 μL of diluted enzymes (50 μg of protein) to initiate the enzymatic reaction (stagger each incubation with 15-s delay intervals) (*see* **Notes 7** and **8**).
3. Incubate the samples at 37°C for 20–30 min (*see* **Note 9**) in a water bath.
4. Add 50 μL of a mixture of 94% acetonitrile/6% glacial acetic acid to stop the enzymatic reaction, and place the incubation tubes on ice.
5. Centrifuge the reaction mixture at 12,000*g* for 4 min.
6. Inject 50–150 μL of the supernatant onto the HPLC column.
7. Run the column at 45–50°C (*see* **Note 10**) at a flow rate of 1 mL/min with a linear gradient from 70% mobile phase A, 30% mobile phase B to 100% B over 20 min. Under these conditions, the retention times are 11 min for 4'-hydroxydiclofenac and 15 min for diclofenac.

8. Prepare blank incubation tubes by adding the complete incubation mixture but with heat-inactivated enzymes. Process the blank incubation tubes per **steps 3–7**.
9. Prepare standards by adding a known amount (e.g., 0, 1, 3, 6, and 12 nmol) of authentic 4'-hydroxydiclofenac metabolite to tubes containing the complete incubation mixture but with heat-inactivated enzymes. Process the standard incubation tubes per **steps 3–7**.
10. Plot a standard curve of the peak area (or peak height) of metabolite standard vs the amount of authentic standard added. Determine the amount of product formation in each unknown sample by linear regression analysis.
11. Calculate diclofenac 4'-hydroxylation activity and express it as nanomoles of product formed/(minute·milligrams of microsomal protein) or as nanomoles of product formed/(minute·nanomoles of total P450).
12. Calculate net enzyme activity by subtracting the activity in the blank from each of the unknown samples (*see* **Note 11**).

## 4. Notes

1. Diclofenac solutions are stable for at least 1 mo when stored at 5°C ***(21)***.
2. Diclofenac 4'-hydroxylation assays can also be carried out with nicotinamide adenine dinucleotide phosphate (NADPH) (e.g., 1 m*M* final concentration) instead of an NADPH-generating system (e.g., $NADP^+$, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase).
3. Prepare only sufficient amounts of dilute microsomes for each experiment. The remainder of the undiluted microsomes can be stored at –80°C for future use ***(22)***.
4. Diclofenac and 4'-hydroxydiclofenac are chromatographed at acidic pH with the carboxylic acid protonated. Separation can also be achieved at slightly alkaline pH (7.4) when the carboxylic acid is ionized, but this may adversely affect column life.
5. Diclofenac and 4'-hydroxydiclofenac can be separated on other brands of $C_{18}$ columns, but this may require adjustment of mobile-phase gradient conditions.
6. A final concentration of 100 µ*M* diclofenac in the incubation mixture is a saturating substrate concentration because the apparent $K_m$ for diclofenac 4'-hydroxylation catalyzed by human liver microsomes is approx 6 µ*M* ***(10)***.
7. With some P450 protein expression systems ***(23)***, it is necessary to reconstitute the recombinant P450 protein with NADPH–cytochrome P450 reductase, cytochrome-$b_5$, and lipid before initiating substrate oxidation. Conduct preliminary experiments to establish the amount of each of these required for optimal catalytic activity.
8. Conduct preliminary experiments to ensure that the assay is linear with respect to microsomal protein concentration.
9. Conduct preliminary experiments to ensure that the assay is linear with respect to incubation time.
10. Column temperature can range from room temperature to 50°C. The use of a controlled, elevated temperature provides greater reproducibility in retention times and lower column back-pressures.
11. Ethanol and dimethyl sulfoxide at a final concentration of 0.3% (v/v) inhibit CYP2C9-catalyzed diclofenac 4'-hydroxylation by 6–10%, whereas no appreciable inhibition occurs with the same concentration of methanol or acetonitrile ***(24)***. However, acetonitrile at final concentrations of ≥0.5% (v/v) stimulates this reaction ***(24,25)***. The stimulation also occurs with tolbutamide as the substrate ***(25,26)***, but not with phenytoin or celecoxib. In fact, inhibition of celecoxib methyl hydroxylation and phenytoin hydroxylation by

acetonitrile occurs at final solvent concentrations of 0.5–5% (v/v) and 5% (v/v), respectively *(25)*.

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# 13

## CYP2C19-Mediated (*S*)-Mephenytoin 4'-Hydroxylation Assayed by High-Performance Liquid Chromatography With Radiometric Detection

**Charles L. Crespi, Thomas K. H. Chang, and David J. Waxman**

**Summary**

A reverse-phase, high-performance liquid chromatography method is described for the quantification of 4'-hydroxymephenytoin formed enzymatically from $^{14}C$-labeled (*S*)-mephenytoin following incubation with cDNA-expressed CYP2C19 or human liver microsomes. Analytical separation is achieved using a $C_{18}$ column developed with a gradient from 10 to 100% methanol, with detection using a scintillation detector. This method is applicable to enzymatic studies for determination of CYP2C19-catalyzed (*S*)-mephenytoin 4'-hydroxylation activity.

**Key Words:** Cytochrome P450; CYP2C19; (*S*)-mephenytoin; 4'-hydroxymephenytoin; (*S*)-mephenytoin 4'-hydroxylation.

### 1. Introduction

CYP2C19 is one of three CYP2C enzymes expressed in human liver ***(1)***. Human liver CYP2C19 is generally expressed at a low level compared with CYP2C8 or CYP2C9 ***(2)***. Considerable interindividual differences occur in hepatic CYP2C19 content ***(3,4)***, primarily owing to a genetic polymorphism in this enzyme ***(5)***. Approximately 12–100% of Asians and 3–5% of Caucasians exhibit the CYP2C19 poor-metabolizer phenotype ***(1)***, which is associated with one of the allelic variants of CYP2C19 (i.e., CYP2C19*2 to CYP2C19*8) ***(5)***. Studies with cDNA-expressed CYP2C19 have identified several drugs as substrates for this polymorphically expressed cytochrome P450 (P450), including omeprazole ***(6,7)***, diazepam ***(8)***, cyclophosphamide ***(2)***, and ifosfamide ***(2)***. Correlational analyses have led to the conclusion that CYP2C19 is a major P450 responsible for (*S*)-mephenytoin 4'-hydroxylation activity in human liver microsomes ***(3,4)***. This is supported by the finding that cDNA-expressed CYP2C19 has a 50- to 100-fold greater turnover number than CYP2C8, CYP2C9, or CYP2C18 for (*S*)-mephenytoin 4'-hydroxylation ***(4)***. The selectivity of

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this reaction for CYP2C19 has been confirmed by immunoinhibition experiments with monoclonal antibody to CYP2C19 ***(9)***. Based on these findings, (*S*)-mephenytoin 4'-hydroxylation activity is frequently used as an enzyme-selective catalytic monitor for human hepatic CYP2C19. Various analytical methods have been developed to measure the enzymatic formation of 4'-hydroxymephenytoin, including isocratic, reverse-phase high-performance liquid chromatography (HPLC) ***(10)***. This chapter describes a method for determining (*S*)-mephenytoin 4'-hydroxylation by gradient, reverse-phase HPLC using $^{14}$C-labeled substrate and radiometric detection.

## 2. Materials

### 2.1. Assay

1. Substrate: [$^{14}$C]-(*S*)-mephenytoin (GE Healthcare Amersham Biosciences, Arlington Heights, IL); (*S*)-mephenytoin (mol wt = 218.3) in methanol (SAFC, formerly Salford, Manchester, UK) (available from BD Biosciences, Discovery Labware, Woburn, MA). Prepare a 10 m*M* (2.18 mg/mL) stock solution of unlabeled (*S*)-mephenytoin in methanol (*see* **Notes 1** and **2**). Evaporate the ethanol from the $^{14}$C-(*S*)-mephenytoin under vacuum or under a stream of nitrogen. Redissolve the residue in methanol at a final concentration of 10 m*M*. Add unlabeled (*S*)-mephenytoin to give a 10 m*M* working solution at 5 mCi/mmol.
2. Metabolite standards: 4'-hydroxymephenytoin (mol wt = 234.3), nirvanol (mol wt = 204.2) (both available from Salford and BD Biosciences, Discovery Labware).
3. Assay buffer: 100 m*M* potassium phosphate, pH 7.4.
4. Cofactor-generating system: 26 m*M* (20 mg/mL) $NADP^+$ (*see* **Note 3**), 66 m*M* (20 mg/mL) D-glucose-6-phosphate, 66 m*M* (13.3 mg/mL) magnesium chloride ($MgCl_2 \cdot 6H_2O$); glucose-6-phosphate dehydrogenase (40 U/mL) in 5 m*M* (1.5 mg/mL) sodium citrate ($C_6H_5O_7Na_3 \cdot 2H_2O$).
5. Enzymes: for example, cDNA-expressed CYP2C19 (BD Biosciences, Discovery Labware) or human liver microsomes can be used. Dilute to a working concentration of 5 mg of protein/mL and keep on ice (*see* **Note 4**).
6. Deproteinizing agent: 100% acetonitrile.

### 2.2. High-Performance Liquid Chromatography

1. Mobile phase A: 10% (v/v) methanol (*see* **Note 5**).
2. Mobile phase B: 100% methanol (*see* **Note 5**).
3. Nucleosil $C_{18}$ column, 4.6 × 250 mm, 5-µm particle size (Sigma-Aldrich, St. Louis, MO) (*see* **Note 6**).
4. Scintillation fluid: Ultima Flo M (Perkin-Elmer, Downers Grove, IL).
5. Detector: radiometric-flow scintillation detector (Perkin-Elmer).

## 3. Methods

1. Add the following to each incubation tube (total incubation volume of 200 µL):
   a. 166 µL of assay buffer.
   b. 2 µL of 10 m*M* (*S*)-mephenytoin (100 µ*M* final concentration) (prepared as described in **Subheading 2.1., item 1**) (*see* **Note 7**).
   c. 10 µL of a solution containing 26 m*M* $NADP^+$, 66 m*M* D-glucose-6-phosphate, and 66 m*M* magnesium chloride.
   d. 2 µL of glucose-6-phosphate dehydrogenase (40 U/mL) in 5 m*M* sodium citrate.

2. Prewarm incubation tubes to 37°C and add 20 µL of diluted enzymes (100 µg of protein) to initiate the enzymatic reaction (stagger each incubation with 15-s delay intervals) (*see* **Notes 8** and **9**).
3. Incubate the samples at 37°C for 45–60 min in a water bath (*see* **Note 10**).
4. Add 50 µL of 100% acetonitrile to stop the enzymatic reaction and place the incubation tubes on ice.
5. Centrifuge the reaction mixture at 12,000*g* for 5 min.
6. Inject 50–150 µL of supernatant onto the HPLC column.
7. Run the column at 45–50°C (*see* **Note 11**) at a flow rate of 1 mL/min with a linear gradient from 80% mobile phase A, 20% mobile phase B to 100% phase B over 10 min, then for a further 10 min with 100% phase B. Under these conditions, retention times are 9 min for 4'-hydroxymephenytoin (*see* **Note 12**) and 16 min for (*S*)-mephenytoin.
8. Prepare blank incubation tubes by adding the complete incubation mixture but with heat-inactivated enzymes. Process the blank incubation tubes per **steps 3–7**.
9. To achieve quantification we use a flow-scintillation detector with scintillation fluid at a flow rate of 3 mL/min with a 0.5-mL flow cell. Determine the counting efficiency using a known quantity of $^{14}C$ with scintillation fluid and HPLC mobile phase in the same proportions as will be present during an analytical run (final volume of 20–50 mL) (*see* **Note 13**). Using the scintillation-fluid pump, fill the flow cell of the detector with this mixture. Measure the radioactivity (cycles per minute [cpm]). The counting efficiency is given by the cpm observed divided by the disintegration per minute (dpm) present in 0.5 mL (the volume of the flow cell). Activity is calculated according to the following equation:

$$\frac{([\text{dpm metabolite}] \times [\text{total volume of incubation + stop addition}])\ /}{([\text{counting efficiency}] \times [2200\ \text{dpm/nCi}] \times [\text{specific activity in nCi/nmol}] \times [\text{mg protein}] \times \text{time} \times [\text{volume injected for analysis}])}$$

10. Calculate (*S*)-mephenytoin 4'-hydroxylase activity and express it as nanomoles of product formed/(minute·milligrams of microsomal protein) or as nanomoles of product formed/(minute·nanomoles of total P450).
11. Calculate net enzyme activity by subtracting the activity in the blank from each of the unknown samples.

## 4. Notes

1. The commercial supply of radiolabeled (*S*)-mephenytoin contains ethanol, which should be evaporated before use. Ethanol and dimethyl sulfoxide inhibit CYP2C19-catalyzed (*S*)-mephenytoin 4-hydroxylation by 20–30% even at a low solvent concentration of 0.1% (v/v) ***(11)***. By contrast, methanol and acetonitrile at a final concentration of 0.3% (v/v) has little or no inhibitory effect ***(11)***.
2. Stock solution of (*S*)-mephenytoin can be stored up to 1 yr at –20°C.
3. (*S*)-mephenytoin 4'-hydroxylase assays can also be carried out with nicotinamide adenine dinucleotide phosphate (NADPH) (e.g., 1 m*M* final concentration) instead of an NADPH-generating system (e.g., $NADP^+$, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase).
4. Prepare only sufficient amounts of dilute microsomes for each experiment. The remainder of the undiluted microsomes can be re-stored at –80°C for future use ***(12)***.
5. Mobile phases containing acetonitrile may be used instead of methanol, but this requires adjustment of mobile-phase gradient conditions. However, the 4'-hydroxymephenytoin peak is often broad and poorly defined with many $C_{18}$ HPLC columns. This problem can

become more pronounced as a column ages with use. The method described here sharpens the 4'-hydroxymephenytoin peak by incorporating a rapid gradient. However, the combination of the methanol/water mobile phase and the need to use a 205-nm wavelength for optimal ultraviolet detection preclude assay quantification by absorbance detection. If absorbance detection is preferred, an acetonitrile/water mobile-phase system should be used. In addition, the use of an alternative HPLC column (Jsphere ODS-L80, cat. no. JL080s04-2546WT; YMC, Wilmington, NC) can provide improved metabolite peak shapes.

6. (*S*)-mephenytoin and 4'-hydroxymephenytoin can be separated on $C_{18}$ columns from other manufacturers but require adjustment of mobile-phase gradient conditions to optimize resolution.
7. In a panel of 14 individual human liver microsome samples, the average apparent $K_m$ for (*S*)-mephenytoin 4'-hydroxylationas approx 100 μ*M* ***(13)***.
8. Conduct preliminary experiments to ensure that the assay is linear with respect to microsomal protein concentration.
9. With CYP2C19 expressed in yeast and some other expression systems, it is necessary to reconstitute the recombinant P450 protein with NADPH–cytochrome P450 reductase, cytochrome-$b_5$, and lipid before inititation of substrate oxidation ***(4)***. Conduct preliminary experiments to establish the amount of each of these required for optimal catalytic activity.
10. Conduct preliminary experiments to ensure that the assay is linear with respect to incubation time.
11. Column temperature can range from room temperature to 50°C. The use of a controlled, elevated temperature provides greater reproducibility in retention times and lower column back-pressures.
12. The other major, identified metabolite from (*S*)-mephenytoin in nirvanol. The HPLC mobility of nirvanol under the specific chromatography conditions should be verified to ensure that it does not cochromatograph with 4'-hydroxymephenytoin.
13. Counting efficiency should be determined in the presence of each of several different mobile-phase solvents because quenching may vary based on solvent composition.

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# 14

# CYP2D6-Dependent Bufuralol 1'-Hydroxylation Assayed by Reverse-Phase Ion-Pair High-Performance Liquid Chromatography With Fluorescence Detection

**Charles L. Crespi, Thomas K. H. Chang, and David J. Waxman**

**Summary**

A reverse-phase, high-performance liquid chromatography method is described for the quantification of 1'-hydroxybufuralol formed enzymatically by the incubation of bufuralol with cDNA-expressed CYP2D6 or human liver microsomes. Analytical separation is achieved using a $C_{18}$ column and a mobile phase consisting of 30% acetonitrile and 2 m*M* perchloric acid, with detection by fluorescence using an excitation wavelength of 252 nm and an emission wavelength of 302 nm. This method is applicable to enzymatic studies for determination of CYP2D6-catalyzed bufuralol 1'-hydroxylation activity.

**Key Words:** Cytochrome P450; CYP2D6; bufuralol; 1'-hydroxybufuralol; bufuralol 1'-hydroxylation.

## 1. Introduction

CYP2D6 is a polymorphically expressed enzyme that metabolizes a large number of clinically useful drugs, including metoprolol, propafenone, haloperidol, dextromethorphan, and codeine *(1)*. Depending on the specific CYP2D6 genotype, an individual may be a poor metabolizer, an intermediate metabolizer, an extensive metabolizer, or an ultrarapid metabolizer *(2)*. CYP2D6 is subject to inhibition by various drugs, including cimetidine *(3,4)* and quinidine *(5)*. Consequently, the extent of CYP2D6-mediated drug clearance may be influenced by other, coadministered therapeutic agents. The competitive inhibition by quinidine is both potent, with a $K_i$ of 3–30 n*M* *(5–7)*, and selective for CYP2D6 *(8,9)*. Quinidine is thus frequently used as an inhibitory chemical probe of this CYP. Diagnostic catalytic monitors of human hepatic CYP2D6 include debrisoquine 4-hydroxylation *(5)*, dextromethorphan *O*-demethylation *(10)*, and bufuralol 1'-hydroxylation activities *(5,7,11,12)*. Advantages of using bufuralol as a CYP2D6-selective substrate include the high sensitivity of the assay owing to the

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highly fluorescent 1'-hydroxybufuralol metabolite and the fact that the use of radiolabeled substrate is not required.

This chapter describes a modification of a reverse-phase ion-pair high-performance liquid chromatography (HPLC) assay *(13)* with fluorescence detection for determination of bufuralol 1'-hydroxylation activity. Methods for other CYP2D6 assays such as debrisoquine 4-hydroxylation *(13)* and dextromethorphan *O*-demethylation *(13,14)* can be found in the cited references.

## 2. Materials

### 2.1. Assay

1. Substrate: (+/–) bufuralol HCl (mol wt = 297.8) (*see* **Note 1**) (SAFC, formerly Salford, Manchester, UK) (available from BD Biosciences, Discovery Labware, Woburn, MA). Prepare a 1 m*M* (0.3 mg/mL) stock solution of bufuralol HCl dissolved in assay buffer (*see* **item 3**; also *see* **Note 2**).
2. Metabolite standard: 1'-hydroxybufuralol maleate (mol wt = 393.4) (SAFC, formerly Salford, available from BD Biosciences, Discovery Labware).
3. Assay buffer: 100 m*M* potassium phosphate, pH 7.4.
4. Cofactor-generating system: 26 m*M* (20 mg/mL) $NADP^+$ (*see* **Note 3**), 66 m*M* (20 mg/mL) D-glucose-6-phosphate, 66 m*M* (13.3 mg/mL) magnesium chloride ($MgCl_2 \cdot 6H_2O$); glucose-6-phosphate dehydrogenase (40 U/mL) in 5 m*M* (1.5 mg/mL) sodium citrate ($C_6H_5O_7Na_3 \cdot 2H_2O$).
5. Enzymes: for example, cDNA-expressed CYP2D6 (BD Biosciences, Discovery Labware) or human liver microsomes can be used. Dilute in assay buffer to a working concentration of 5 mg of protein/mL and keep on ice (*see* **Note 4**).
6. Deproteinizing agent: 70% (v/v) perchloric acid.

### 2.2. High-Performance Liquid Chromatography

1. Mobile phase: 30% (v/v) acetonitrile, 2 m*M* perchloric acid (*see* **Note 5**).
2. Nucleosil $C_{18}$ column, 4.6 × 250 mm, 5-µ*M* particle size (Sigma-Aldrich, St. Louis, MO) (*see* **Note 6**).
3. Fluorescence detector with an excitation wavelength of 252 nm, an emission wavelength of 302 nm, and a slit width of 5–10 nm.

## 3. Methods

1. Add the following to each incubation tube (total incubation volume of 200 µL):
   a. 163 µL of assay buffer.
   b. 5 µL of 1 m*M* (+/–) bufuralol (25 µ*M* final concentration; *see* **Note 7**).
   c. 10 µL of a solution containing 26 m*M* $NADP^+$, 66 m*M* D-glucose-6-phosphate, and 66 m*M* magnesium chloride ($MgCl_2 \cdot 6H_2O$).
   d. 2 µL of glucose-6-phosphate dehydrogenase (40 U/mL) in 5 m*M* sodium citrate.
2. Prewarm incubation tubes to 37°C and add 20 µL of diluted enzymes (100 µg of protein) to initiate the enzymatic reaction (stagger each incubation with 15-s delay intervals) (*see* **Notes 8** and **9**).
3. Incubate the samples at 37°C for 10 min in a water bath (*see* **Note 10**).
4. Add 20 µL of 70% (v/v) perchloric acid to stop the enzymatic reaction and place the incubation tubes on ice.

5. Centrifuge the reaction mixture at 12,000*g* for 4 min.
6. Inject 10–50 μL of supernatant onto the HPLC column.
7. Run the column at 45–50°C (*see* **Note 11**) with 30% (v/v) acetonitrile, 2 m*M* perchloric acid at a flow rate of 1 mL/min. Under these conditions, retention times are 6 min for 1'-hydroxybufuralol and 20 min for bufuralol. Monitor the fluorescence (excitation wavelength of 252 nm, emission wavelength of 302 nm).
8. Prepare blank incubation tubes by adding the complete incubation mixture but with heat-inactivated enzymes. Process the incubation tubes per **steps 3–7**.
9. Prepare standards by adding a known amount (e.g., 0, 0.05, 0.1, 0.2, 0.5, and 1 nmol) of authentic 1'-hydroxybufuralol metabolite to tubes containing the complete incubation mixture but with heat-inactivated enzymes. Process the incubation tubes containing the standards per **steps 3–7**.
10. Plot a standard curve of peak height (or peak area) of metabolite standard vs the amount of authentic metabolite added. Determine the amount of product formation in each unknown and blank sample.
11. Calculate bufuralol 1'-hydroxylation activity and express it as nanomoles of product formed/(minute·milligrams of microsomal protein) or as nanomoles of product formed/(minute·nanomoles of total P450).
12. Calculate net enzyme activity by subtracting the activity in the blank from each of the unknown samples (*see* **Notes 12** and **13**).

## 4. Notes

1. The metabolism of bufuralol by CYP2D6 is enantioselective. The apparent $k_{cat}$ values for (+) bufuralol 1'-hydroxylation and (–) bufuralol 1'-hydroxylation catalyzed by purified, reconstituted CYP2D6 are 3.7 nmol/(min·nmol of P450) (i.e., 3.7/min for the [+] substrate), and 0.6 nmol/(min·nmol of cytochrome P450 [P450]) (i.e., 0.6/min for the [–] substrate) ***(5)***.
2. Bufuralol stock solutions may be stored at –20°C for up to 2 yr.
3. Bufuralol 1'-hydroxylation assays can also be carried out with nicotinamide adenine dinucleotide phosphate (NADPH) (e.g., 1 m*M* final concentration) instead of an NADPH-generating system (e.g., $NADP^+$, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase).
4. Prepare only sufficient amounts of dilute microsomes for each experiment. The remainder of the undiluted microsomes can be re-stored at –80°C for future use ***(15)***.
5. The mobile phase must include perchloric acid because the metabolite chromatographs as an ion pair.
6. Bufuralol and 1'-hydroxybufuralol can be separated by $C_{18}$ columns from other manufacturers but may require adjustment of the acetonitrile content of the mobile phase.
7. Apparent $K_m$ values reported for bufuralol 1'-hydroxylation catalyzed by cDNA-expressed CYP2D6 range from 5 to 40 μ*M* ***(16–19)***. A substrate concentration of 25 μ*M* is recommended when bufuralol 1'-hydroxylation activity is used as a marker for hepatic CYP2D6 because this P450 accounts for nearly all of bufuralol 1'-hydroxylation activity in human liver microsomes when the assay is carried out at this substrate concentration ***(17)***. However, as the substrate concentration increases, the contribution of CYP2D6 to the microsomal activity decreases because of the increased role of a higher-$K_m$ form such as CYP1A2. This is consistent with the reported nonlinear Eadie-Hofstee plot for bufuralol 1'-hydroxylation catalyzed by microsomes isolated from livers associated with the CYP2D6 extensive metabolizer phenotype ***(13)***. Quinidine (1 μ*M* final concentration from

a 1 m*M* stock solution prepared in assay buffer) can be added to confirm the specificity of catalysis by CYP2D6.

8. With some P450 expression systems, it is necessary to reconstitute the recombinant P450 protein with NADPH–cytochrome P450 reductase, cytochrome-$b_5$, and lipid before initiating substrate oxidation (*17,18*). Conduct preliminary experiments to establish the amount of each of these required for optimal catalytic activity.
9. Conduct preliminary experiments to ensure that the assay is linear with respect to microsomal protein concentration.
10. Conduct preliminary experiments to ensure that the assay is linear with respect to incubation time.
11. Column temperature can range from room temperature to 50°C. The use of a controlled, elevated temperature provides greater reproducibility in retention times and lower column back-pressures.
12. In a panel of 60 human liver microsomes, bufuralol 1'-hydroxylation activity ranged from 0 to 0.04 nmol/(min·mg of protein) (*17*).
13. Acetonitrile at a final concentration of 0.3% (v/v) does not appreciably inhibit CYP2D6-catalyzed bufuralol 1'-hydroxylation activity (*20*). However, substantial inhibition (25–40%) occurs with ethanol and dimethyl sulfoxide (*20*).

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# 15

# Spectrophotometric Analysis of Human CYP2E1-Catalyzed *p*-Nitrophenol Hydroxylation

**Thomas K. H. Chang, Charles L. Crespi, and David J. Waxman**

**Summary**

The cytochrome P450 enzyme CYP2E1 catalyzes the oxidative metabolism of many solvents and other small organic molecules. A spectrophotometric method is described for determination of CYP2E1 activity by monitoring the formation of *p*-nitrocatechol from *p*-nitrophenol by cDNA-expressed CYP2E1 or isolated liver microsomes. The enzymatic product, *p*-nitrocatechol, is assayed at 535 nm after acidification of the reaction mixture with trichloroacetic acid followed by neutralization using 2 *M* NaOH. This method is applicable to enzymatic studies for determination of P450-catalyzed *p*-nitrophenol hydroxylation activity.

**Key Words:** Cytochrome P450; CYP2E1; *p*-nitrophenol; *p*-nitrocatechol; *p*-nitrophenol hydroxylation.

## 1. Introduction

CYP2E1 is expressed in adult *(1)* and fetal *(2)* human liver in addition to extrahepatic tissues such as lung and placenta *(3)*. Treatment of primary cultures of human hepatocytes with ethanol induces CYP2E1 protein *(4)*, and this is consistent with the finding that hepatic CYP2E1 protein *(5)* and mRNA *(6)* levels are increased in individuals with alcoholism. Although only a few drugs (e.g., acetaminophen *[7]*) have been identified as substrates for CYP2E1, many low molecular weight procarcinogens are activated by this cytochrome P450 (P450) *(8,9)*. Chlorzoxazone 6-hydroxylation *(10–12)*, *N*-nitrosodimethylamine *N*-demethylation *(11,13,14)*, and *p*-nitrophenol hydroxylation *(12,14)* can be used to measure the catalytic activity of cDNA-expressed CYP2E1. Each of these activities can also be used as an enzyme-selective catalytic monitor for human hepatic CYP2E1 (*see* **Notes 1** and **2**), as demonstrated by experiments with inhibitory anti-CYP2E1 antibodies and CYP2E1-selective chemical inhibitors *(8,15–18)*. Lauric acid 11-hydroxylation has been identified as an alternative probe for human hepatic CY2E1 *(19)*. *p*-Nitrophenol hydroxylation can easily be assayed by monitoring the formation of *p*-nitrocatechol in a simple spectrophotometric

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assay. High-performance liquid chromatography assays have also been developed to quantify the *p*-nitrocatechol metabolite ***(20,21)***.

This chapter describes a modification of a spectrophotometric method ***(22)*** for determination of *p*-nitrophenol hydroxylation activity. Methods for other CYP2E1 assays such as chlorzoxazone 6-hydroxylation ***(23)***, *N*-nitrosodimethylamine *N*-demethylation ***(24)***, and lauric acid 11-hydroxylation ***(19)*** can be found in the cited references.

## 2. Materials

1. Assay buffer: 50 m*M* potassium phosphate, pH 7.4.
2. Substrate: *p*-nitrophenol (mol wt = 139.1; *see* **Note 3**) (Sigma-Aldrich, St. Louis, MO). Prepare a 5 m*M* (0.70 mg/mL) stock solution dissolved in assay buffer (*see* **Note 4**).
3. Metabolite standard: *p*-nitrocatechol (mol wt = 155.1; *see* **Notes 3** and **4**) (Sigma-Aldrich).
4. Cofactor-generating system: 26 m*M* (20 mg/mL) $NADP^+$ (*see* **Note 5**), 66 m*M* (20 mg/mL) D-glucose-6-phosphate, 66 m*M* (13.3 mg/mL) magnesium chloride ($MgCl_2 \cdot 6H_2O$); glucose-6-phosphate dehydrogenase (40 U/mL) in 5 m*M* (1.5 mg/mL) sodium citrate ($C_6H_5O_7Na_3 \cdot 2H_2O$).
5. Enzymes: for example, cDNA-expressed CYP2E1 (BD Biosciences, Discovery Labware, Woburn, MA) or human liver microsomes can be used. Dilute in assay buffer to a working concentration of 5 mg of protein/mL and keep on ice (*see* **Note 6**).
6. Deproteinizing agent: 20% (v/v) trichloroacetic acid (TCA).
7. 2 *M* NaOH.
8. Vortex mixer, spectrophotometer, and water bath.

## 3. Methods

1. Add the following to each incubation tube (total incubation volume of 500 µL):
   a. 440 µL of assay buffer.
   b. 10 µL of 5 m*M* *p*-nitrophenol (100 µ*M* final concentration; *see* **Note 7**).
   c. 25 µL of a solution containing 26 m*M* $NADP^+$, 66 m*M* D-glucose-6-phosphate, and 66 m*M* magnesium chloride.
   d. 5 µL of glucose-6-phosphate dehydrogenase (40 U/mL) in 5 m*M* sodium citrate.
2. Prewarm incubation tubes to 37°C, and add 20 µL of diluted enzymes (100 µg of protein) to initiate the enzymatic reaction (stagger each incubation with 15-s delay intervals) (*see* **Notes 8** and **9**).
3. Incubate the samples at 37°C for 30 min in a water bath (*see* **Note 10**).
4. Add 100 µL of 20% (v/v) TCA to stop the enzymatic reaction and place the incubation tubes on ice.
5. Centrifuge the reaction mixture at 10,000*g* for 5 min.
6. Transfer 0.5 mL of the supernatant to a clean test tube containing 0.25 mL of 2 *M* NaOH and vortex.
7. Measure the absorbance at 535 nm with a spectrophotometer (*see* **Note 11**).
8. Prepare blank incubation tubes by adding the complete incubation mixture but with heat-inactivated enzymes. Process the blank incubation tubes per **steps 3–7**.
9. Prepare standards by adding a known amount (e.g., 0, 1, 2, 5, 10, and 20 nmol) of authentic *p*-nitrocatechol metabolite (*see* **Note 4**) to the tubes containing the complete incubation mixture but with heat-inactivated enzymes. Process the standard tubes per **steps 3–7**.

10. Calculate the net absorbance of each unknown sample and standard by subtracting the absorbance of the blank tubes from that of the unknown sample or standard.
11. Plot a standard curve of net aborbance vs the amount of authentic *p*-nitrocatechol standard, and determine the amount of product formed in each unknown sample by linear regression analysis.
12. Calculate *p*-nitrophenol hydroxylation activity and express it as nanomoles of product formed/(minute·milligrams of microsomal protein) or as nanomoles of product formed/(minute·nanomoles of total P450) (*see* **Note 12**).

## 4. Notes

1. Exercise caution when using hepatic microsomal chlorzoxazone 6-hydroxylation activity as a diagnostic catalytic marker for human CYP2E1 because of the reported contribution of CYP3A to this activity in human hepatic microsomes ***(25)***. In addition to cDNA-expressed CYP2E1, recombinant human CYP1A1, CYP3A4, and CYP2D6 have significant catalytic activity toward chlorzoxazone 6-hydroxylation ***(25,26)***. A high concentration (e.g., 500 µ*M*) of chlorzoxazone should be used in order to minimize the contribution from the lower-$K_m$ enzymes ***(27)***. At a substrate concentration of 500 µ*M*, CYP2E1 accounts for only 65% of the chlorzoxazone 6-hydroxylation activity in human liver microsomes, as indicated by immunoinhibition experiments with a monoclonal antibody to CYP2E1 ***(28)***.
2. Exercise caution when using *p*-nitrophenol hydroxylation activity as a diagnostic probe for CYP2E1 in human liver microsomes, because CYP3A4 and CYP2A6 may potentially contribute to this activity ***(29)***.
3. *p*-Nitrophenol and *p*-nitrocatechol are light sensitive.
4. Stock solutions of *p*-nitrophenol and *p*-nitrocatechol can be stored for up to 1 yr at –20°C.
5. *p*-Nitrophenol hydroxylation assays can also be carried out with nicotinamide adenine dinucleotide phosphate (NADPH) ***(30)*** instead of an NADPH-generating system (e.g., $NADP^+$, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase).
6. Prepare only sufficient amounts of diluted human liver microsomes for each experiment. The remainder of the undiluted microsomes can be stored at –80°C for future use ***(31)***.
7. A substrate concentration of 100 µ*M* is suggested because the apparent $K_m$ for *p*-nitrophenol hydroxylation by cDNA-expressed CYP2E1 and by human liver microsomes is approx 20–30 µ*M* ***(17)*** and substrate inhibition is observed at >200 µ*M* *p*-nitrophenol ***(22)***.
8. With some P450 protein expression systems, it is necessary to reconstitute the recombinant P450 protein with NADPH–cytochrome P450 reductase, cytochrome-$b_5$, and lipid before initiating substrate oxidation ***(12)***. Conduct preliminary experiments to establish the amount of each of these required for optimal catalytic activity.
9. Conduct preliminary experiments to ensure that the assay is linear with respect to microsomal protein concentration.
10. Conduct preliminary experiments to ensure that the assay is linear with respect to incubation time.
11. Read the absorbance immediately on mixing with 2 *M* NaOH. Over time, the alkaline pH increases the background in the assay.
12. In a panel of 10 individual human liver microsome samples, *p*-nitrophenol hydroxylation activity ranged from 0.25 to 3.3 nmol/(min·mg of protein) ***(30)***.

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# 16

# Thin-Layer Chromatography Analysis of Human CYP3A-Catalyzed Testosterone 6β-Hydroxylation

**David J. Waxman and Thomas K. H. Chang**

**Summary**

Testosterone and other steroid hormones have been studied as prototypic examples of endogenous substrates for hepatic cytochrome P450 (P450) enzymes. CYP3A enzymes from various species, including human, metabolize testosterone by a 6β-hydroxylation reaction, which is unique to this P450 subfamily. A thin-layer chromatographic method is described for the determination of 6β-hydroxytestosterone formed enzymatically by incubation of [$^{14}$C]-testosterone with cDNA-expressed CYP3A enzymes or liver microsomes. $^{14}$C-labeled enzymatic products are applied to silica gel thin-layer plates, which are developed sequentially with methylene chloride:acetone (80:20) followed by chloroform, ethyl acetate, and absolute ethanol (80:20:14). Metabolite quantification is performed by autoradiography and liquid scintillation counting. This method is applicable to enzymatic studies for the determination of CYP3A-dependent testosterone 6β-hydroxylation activity in both human and animal liver microsomes.

**Key Words:** Cytochrome P450; CYP3A; CYP3A4; CYP3A5; CYP3A7; CYP3A43; testosterone; 6β-hydroxytestosterone; testosterone 6β-hydroxylation.

## 1. Introduction

The human CYP3A subfamily contains four forms: CYP3A4, CYP3A5, CYP3A7, and CYP3A43 ***(1)***. CYP3A4, CYP3A5, and CYP3A43 are expressed in adult human liver ***(2)***, whereas CYP3A7 is present only in human fetal liver ***(3)***. CYP3A4 is present in all adult human livers and is inducible by drugs such as rifampin and dexamethasone ***(4–6)***. By contrast, CYP3A5 is expressed in only approx 10–30% of liver samples ***(7,8)*** and does not respond to typical CYP3A inducers ***(5,6)***. Of the three CYP3A forms present in adult human liver, CYP3A4 is the most abundant, accounting for 95% of all the CYP3A mRNA ***(9)***. By comparison, only 2% of the total CYP3A protein content is CYP3A5 ***(8)***, and the ratio of CYP3A43 mRNA/CYP3A4 mRNA is 0.001 ***(10)***. Collectively, CYP3A enzymes account for an estimated 30% of total human cytochrome P450 (P450) content in adult liver ***(11)***, although large interindividual

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differences exist in hepatic CYP3A content. Triacetyloleandomycin *(12,13)* and gestodene *(13)* are CYP3A-selective chemical inhibitors. Many commonly used drugs are substrates for CYP3A, including erythromycin *(14)*, nifedipine *(15)*, and midazolam *(16)*. Immunoinhibition experiments with CYP3A subfamily–specific antibodies have established several microsomal enzyme activities as useful catalytic monitors for hepatic CYP3A. These include midazolam 1'-hydroxylation *(17)*, nifedipine oxidation *(15,18)*, and testosterone 6β-hydroxylation *(19–22)*. Testosterone 6β-hydroxylation activity has a high specificity for hepatic CYP3A4, as indicated in a study in which an inhibitory antipeptide antibody against CYP3A4, which did not crossreact with cDNA-expressed CYP3A5 and did not inhibit cDNA-expressed CYP3A5-catalyzed testosterone 6β-hydroxylation, blocked virtually all of the testosterone 6β-hydroxylation activity in human liver microsomes *(23)*. This is consistent with the following earlier findings:

1. CYP3A4 is expressed at a much higher level than CYP3A5 in human liver *(24)*.
2. cDNA-expressed CYP3A4 is much more active than cDNA-expressed CYP3A5 in testosterone 6β-hydroxylation *(25,26)*.
3. cDNA-expressed CYP3A5 exhibits an approx 10-fold greater apparent $K_m$ than cDNA-expressed CYP3A4 in testosterone 6β-hydroxylation *(26)*.

Several analytical methods are available for determining testosterone 6β-hydroxylation, including isocratic *(27)* and gradient *(28,29)* high-performance liquid chromatography (HPLC) methods, and HPLC-tandem mass spectrometry *(30)*. This chapter describes a radiometric thin-layer chromatography (TLC) assay for determination of testosterone 6β-hydroxylation activity. As indicated in **Subheading 4.**, this assay method can be used to determine P450-catalyzed 6β-hydroxylation of related steroids, such as androstenedione and progesterones and to assay testosterone hydroxylation activity at positions other than C-6β. Methods for other CYP3A assays such as nifedipine oxidation *(15)* and midazolam 1'-hydroxylation *(16)* can be found in the cited references.

## 2. Materials

1. Substrate: [4-$^{14}$C]testosterone, 50–62 mCi/mmol, 50 mCi/mL (Amersham, Arlington Heights, IL); testosterone (mol wt = 288.4) (Sigma-Aldrich, St. Louis, MO). Dilute [4-$^{14}$C]testosterone with unlabeled testosterone dissolved in toluene to give a working solution at 5–7 mCi/mmol (4000–6000 cpm/μL).
2. Metabolite standards:
   a. 2α-Hydroxytestosterone (Sigma-Aldrich).
   b. 2β-Hydroxytestosterone (Sigma-Aldrich).
   c. 6α-Hydroxytestosterone (Sigma-Aldrich).
   d. 6β-Hydroxytestosterone (Sigma-Aldrich).
   e. 7α-Hydroxytestosterone (Steraloids, Wilton, NH).
   f. 11β-Hydroxytestosterone (Sigma-Aldrich).
   g. 14α-Hydroxytestosterone (Sigma-Aldrich).
   h. 15α-Hydroxytestosterone (Sigma-Aldrich).
   i. 15β-Hydroxytestosterone (Searle, Skokie, IL).

   j. 16α-Hydroxytestosterone (Sigma-Aldrich).
   k. 16β-Hydroxytestosterone (Steraloids).
   l. 19-Hydroxytestosterone (Steraloids).
3. Assay buffer: 100 m*M* HEPES, pH 7.4, containing 0.1 m*M* EDTA.
4. Cofactor: 10 m*M* (8.3 mg/mL) nicotinamide adenine dinucleotide phosphate (NADPH) stock solution; prepare fresh and keep on ice (*see* **Notes 1** and **2**).
5. Enzymes: for example, cDNA-expressed CYP3A4, CYP3A5 (BD Biosciences, Discovery Labware) or human liver microsomes. Dilute in assay buffer to a working concentration of 1.5 mg of protein/mL and keep on ice (*see* **Note 3**).
6. Extraction solvent: ethyl acetate.
7. TLC plates: aluminum-backed silica gel 60 F-254 plates, 20 × 20 cm (EM Separations Technology, Gibbstown, NJ).
8. Glass TLC tank, approx 10 × 30 cm, 25 cm tall.
9. TLC solvent mixtures (prepare before use):
   a. 80 mL of methylene chloride and 20 mL of acetone.
   b. 80 mL of chloroform, 20 mL of ethyl acetate, and 14 mL of absolute ethanol.
   c. 80 mL of dichloromethane and 20 mL of acetone.
10. Whatman 3MM chromatography paper.
11. X-ray film: Kodak X-OMAT AR.
12. X-ray film holder.
13. Scintillation cocktail: Betafluor (National Diagnostics, Manville, NJ) or equivalent.
14. Equipment including a liquid scintillation counter and water bath.

## 3. Methods

### 3.1. Enzymatic Reaction

1. To each incubation tube (13 × 100 mm test tube), add 10 nmol of [4-$^{14}$C]testosterone at 5–7 mCi/mmol (50 μ*M* steroid in a final assay volume of 0.2 mL), and evaporate the solvent under a gentle stream of nitrogen.
2. Add the following to each incubation tube (total incubation volume of 200 μL): 160 μL of assay buffer and 20 μL of diluted enzymes (30 μg of protein) (*see* **Notes 4** and **5**).
3. Prewarm the incubation tubes to 37°C and add 20 μL of 10 m*M* NADPH (1 m*M* final concentration) to initiate the enzymatic reaction (stagger each incubation with 15-s delay intervals).
4. Incubate the samples at 37°C for 10–20 min in a shaking water bath (*see* **Note 6**).
5. Add 1 mL of ice-cold ethyl acetate to stop the enzymatic reaction, vortex briefly, and place the incubation tubes on ice until all the samples have been collected.
6. Vortex for 30 s (to extract with ethyl acetate).
7. Centrifuge the reaction mixture at 3000*g* for 5 min.
8. Transfer the organic phase (upper layer) to a clean test tube.
9. To the aqueous phase add 1 mL of ethyl acetate and repeat **steps 6** and **7**.
10. Combine the organic extracts and dry under a gentle stream of nitrogen.
11. Process the blank incubation tubes containing the complete incubation mixture but with heat-inactivated enzymes as per **steps 4–10**.

### 3.2. TLC and Autoradiography

1. Activate aluminum-backed, silica gel TLC plates by heating at 100°C for 10–15 min. Allow to cool for 5 min (*see* **Note 7**).

2. Add solvent mixture (80 mL of methylene chloride and 20 mL of acetone) to a glass TLC tank. Add one sheet of Whatman 3MM chromatography paper to the tank to help saturate the air with solvent vapors. Equilibrate for at least 20 min.
3. Reconstitute the dried organic extracts containing $^{14}C$-labeled enzymatic products (*see* **Subheading 3.1., step 10**) by adding 30 µL of ethyl acetate to each assay tube.
4. Apply the reconstituted enzymatic products with a 10-µL micropipet in several portions as individual spots. Each spot should be ≤2 mm in diameter and is applied at the origin on a line drawn 2 cm up from the bottom of a heat-activated TLC plate (*see* **Note 8**).
5. Repeat **step 3** to wash out the tubes and increase the recovery of $^{14}C$-labeled metabolites.
6. Repeat **step 4**.
7. Place the TLC plates in the solvent-preequilibrated TLC tank (*see* **step 2**).
8. Remove the plates from the TLC tank when the solvent front is approx 2 cm from the top. Air-dry the plates for approx 10 min.
9. Place the TLC plates in a second TLC tank preequilibrated with solvent mixture (80 mL of chloroform, 20 mL of ethyl acetate, and 14 mL of absolute ethanol) (*see* **Note 9**).
10. Repeat **step 8**.
11. Mark the corners of each TLC plate with three to four small spots drawn in a characteristic pattern using either a fluorescent marker or radioactive ink ("hot ink spots"). These spots will expose the X-ray film (*see* **step 12**) in a characteristic pattern that will then be used to align the film with the TLC plates for quantification (*see* **Subheading 3.3.**).
12. Expose the TLC plates to X-ray film in a film holder overnight at room temperature.
13. Develop the X-ray film to visualize the pattern of substrate and metabolites (*see* **Notes 10** and **11**).

### 3.3. Metabolite Quantification

1. Align the "hot ink spots" on the corners of the X-ray film with those seen on the TLC plate.
2. Identify the location of each metabolite on the TLC plate by drawing a rectangular grid in pencil, and marking the regions of the plate that encompass each individual $^{14}C$-labeled spot visible on the X-ray film.
3. Use scissors to cut out each of these marked rectangular areas from the TLC plate.
4. Place each rectangular piece of the aluminum-backed plate in a labeled 20-mL scintillation vial containing 7 mL of Betafluor scintillation cocktail with the silica gel side facing up (*see* **Note 12**).
5. Determine the counts per minute (cpm) in each vial using a liquid scintillation counter (*see* **Note 13**).
6. Determine the background cpm for each region of the TLC plate by counting the corresponding silica gel pieces cut from a control (no microsomes or no NADPH blank) incubation lane.
7. Calculate the net cpm by subtracting the cpm of the blank from that of the unknown.
8. Calculate the enzyme activity (nmol/[min·mg of protein]) for each hydroxytestosterone metabolite as follows (*see* **Notes 14–16**):

$$\text{enzyme activity} = \frac{\text{cpm of product} \times \text{nmol of substrate}}{(\text{total cpm of all spots in a lane}) \times (\text{mg of protein}) \times (\text{incubation time})}$$

9. Alternatively, if the total P450 content of the sample has been determined, testosterone 6β-hydroxylation activity may be expressed as nanomoles of product formed/(minute · nanomoles of total P450).

### *3.4. Identification of 6β-Hydroxytestosterone Metabolite*

1. Dissolve authentic 6β-hydroxytestosterone in ethyl acetate at a concentration of approx 0.3–1 mg/mL.
2. Activate an aluminum-backed, silica-gel TLC plate by heating at 100°C for 10–15 min. Allow to cool for 5 min.
3. Apply the authentic monohydroxytestosterone metabolite onto a heat-activated TLC plate as per **Subheading 3.2., step 4**.
4. Directly on top of a spot containing authentic metabolite applied in **step 3**, apply a portion of the $^{14}$C-labeled testosterone metabolites generated from **Subheading 3.1.** Alternatively, the $^{14}$C-labeled metabolite can be mixed with the unlabeled metabolite standard and then the mixture applied to the TLC plate.
5. Carry out TLC analysis as per **Subheading 3.2., steps 7–13**.
6. After developing the X-ray film, visualize the location of the authentic 6β-hydroxytestosterone with an ultraviolet (UV) lamp, and lightly outline its location on the TLC plate using a pencil. The unlabeled hydroxytesterone is visible on the TLC plate as a dark spot on a background of green fluorescence.
7. Align the "hot ink" spots on the corners of the X-ray film with those on the TLC plate and overlay the X-ray film precisely over the TLC plate. Coincidence of the $^{14}$C-labeled X-ray film spot with the unlabeled UV-active hydroxytestosterone metabolite provides supporting evidence for product identification.
8. To further confirm product identification, repeat **steps 1–7** using a different TLC solvent system: two sequential developments in solvent mixture (80 mL of dichloromethane and 20 mL of acetone).
9. Repeat **steps 1–7**, but perform the TLC with a third solvent system: two sequential developments in solvent mixture (80 mL of chloroform, 20 mL of ethyl acetate, and 14 mL of absolute ethanol). An unknown metabolite is deemed to be "identified" as 6β-hydroxytestosterone if cochromatography of radiolabeled metabolite with unlabeled authentic 6β-hydroxytestosterone standard is evident in each of the three TLC solvent systems.
10. To rule out the possibility that the unknown metabolite is a metabolite other than 6β-hydroxytestosterone, repeat **steps 1–9** but with unlabeled authentic monohydroxytestosterone metabolites such as those listed in **Subheading 2., item 2**.

## 4. Notes

1. Prepare fresh solutions of NADPH for each experiment. Note that NADPH is light- and pH sensitive.
2. Testosterone 6β-hydroxylation assays can also be carried out with an NADPH-generating system (e.g., $NADP^+$, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) in place of NADPH ***(31)***.
3. Prepare only sufficient amounts of dilute microsomes for each experiment. The remainder of the undiluted microsomes can be stored at –80°C for future use ***(32)***.
4. Conduct preliminary experiments to ensure that the assay is linear with respect to microsomal protein concentration.
5. Recombinant CYP3A4 enzymes, such as those produced in a yeast expression system, need to be reconstituted with exogenous NADPH–cytochrome P450 reductase, cytochrome-$b_5$, and dilauroylphosphatidylcholine (DLPC) prior to initiation of substrate oxidation ***(33,34)***. Preliminary experiments will be required to determine the optimal amounts of NADPH-cytochrome P450 reductase, cytochrome-$b_5$, and DLPC to be used. However,

a high level of catalytic activity can be achieved by coexpression of recombinant CYP3A4 with NADPH–cytochrome P450 reductase and/or cytochrome-$b_5$ (*35,36*).

6. Conduct preliminary experiments to ensure that the assay is linear with respect to incubation time.
7. To prevent curling of the TLC plates during heating at 100°C, cover the edges of each plate with aluminum foil.
8. To minimize "end lane" effects, do not apply samples within 2 to 3 cm from the edge of each TLC plate.
9. The assay described here can be used to determine human liver microsomal steroid 6β-hydroxylase activity for two related steroids, androstenedione and progesterone, by modifying the TLC solvent system. Androstenedione metabolites are resolved by two sequential developments of the TLC plates in chloroform/ethyl acetate (1:2 [v/v]), and progesterone metabolites are resolved by TLC using ethyl acetate/*n*-hexane/acetic acid (19:5:1 [v/v/v]) (*19,37*).
10. Rapid and sensitive quantification of the $^{14}$C-labeled metabolites can also be achieved using a phosphorimager instrument (Molecular Imager from Bio-Rad, Hercules, CA; or STORM 840 Imager from Molecular Dynamics, Sunnyvale, CA), thus eliminating the X-ray film exposure, TLC cutting, and liquid scintillation counting steps.
11. Although 6β-hydroxytestosterone is the major hydroxytestosterone metabolite formed by human liver microsomes, several other hydroxytestosterone metabolites are also formed (*19*). These metabolites can also be determined alongside 6β-hydroxytestosterone in the same TLC assay.
12. Significant quenching (approx 25% decrease in cpm) will occur if the aluminum-backed side of the cut TLC piece faces up during liquid scintillation counting.
13. The overall recovery of cpm initially added to each incubation tube is 65–85%.
14. Duplicate activity analyses generally agree within 5–7%.
15. In a panel of six human liver microsomes, this activity ranged from 0.09 to 1.48 nmol/(min·mg of microsomal protein) (*19*), whereas in another panel of 10 microsome samples, it ranged from 0.56 to 8.41 nmol/(min·mg of microsomal protein) (*7*).
16. Dimethyl sulfoxide at a final concentration of 0.1% (v/v) inhibits testosterone 6β-hydroxylation activity by approx 25% (*38*). By contrast, little or no inhibition occurs with methanol, ethanol, or acetonitrile at a final concentration of up to 0.5% (v/v) (*38,39*).

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# 17

## Determination of CYP4A11-Catalyzed Lauric Acid 12-Hydroxylation by High-Performance Liquid Chromatography With Radiometric Detection

**Charles L. Crespi, Thomas K. H. Chang, and David J. Waxman**

### Summary

Lauric acid serves as an endogenous substrate for the cytochrome P450 enzyme CYP4A11. A reverse-phase, high-performance liquid chromatography method is described for the quantification of 12-hydroxylauric acid formed enzymatically by incubation of $^{14}$C-labeled lauric acid with cDNA-expressed CYP4A11 or human liver microsomes. Analytical separation is achieved using a $C_{18}$ column and a gradient of 30% acetonitrile and 2 m*M* perchloric acid to 100% methanol, using a detection scintillation counter. This method is applicable to enzymatic studies for determination of lauric acid 12-hydroxylation activity.

**Key Words:** Cytochrome P450; CYP4A11; lauric acid; 12-hydroxylauric acid; lauric acid 12-hydroxylation.

## 1. Introduction

The human *CYP4A11* gene encodes a cytochrome P450 (P450) protein that has been isolated from human kidney ***(1,2)*** and liver ***(3)*** and purified to apparent homogeneity. CYP4A11 mRNA is present in greater abundance in kidney than in liver, whereas it is absent in lung ***(3)***. Various fatty acids, including lauric acid ***(2–4)***, are substrates for recombinant CYP4A11. Experiments with a panel of individual cDNA-expressed P450 enzymes indicate that CYP4A11 is a major lauric acid 12-hydroxylase ***(5)***. Immunoinhibition experiments with heterologous and anti-human CYP4A11 antibodies have further established that CYP4A11 contributes to a major fraction of the lauric acid 12-hydroxylase activity in human liver microsomes ***(3,5,6)***. Accordingly, microsomal lauric acid 12-hydroxylation activity is used as a marker for human hepatic CYP4A11. Although CYP4A11 is a major lauric acid 12-hydroxylase in human liver, lauric acid 11-hydroxylation in this tissue is catalyzed by CYP2E1 and not CYP4A11 ***(7,8)***. Several analytical methods are available for quantification of 12-

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hydroxylauric acid, including the use of nonradioactive lauric acid as the substrate by a high-performance liquid chromatography (HPLC) assay with fluorescence detection (*9,10*). This chapter describes a radiometric HPLC assay for determination of lauric acid 12-hydroxylation activity.

## 2. Materials

### 2.1. Assay

1. Assay buffer: 100 m*M* Tris-HCl, pH 7.5.
2. Substrate: [1-$^{14}$C]lauric acid (50–62 mCi/mmol, 50 mCi/mL) (GE Healthcare Amersham Biosciences, Arlington Heights, IL); lauric acid (sodium salt, mol wt = 222.3) (Sigma-Aldrich, St. Louis, MO). Prepare unlabeled lauric acid stock (5 m*M* in 100 m*M* Tris-HCl, pH 7.5). Evaporate hexane solvent from [$^{14}$C]lauric acid. Dissolve in a small volume of 100 m*M* Tris-HCl, pH 7.5. Dilute [1-$^{14}$C]lauric acid with unlabeled lauric acid dissolved in 100 m*M* Tris-HCl, pH 7.5, to give a working solution at 5 mCi/mmol (1 m*M*).
3. Cofactor-generating system: 26 m*M* (20 mg/mL) $NADP^+$, 66 m*M* (20 mg/mL) D-glucose-6-phosphate, and 66 m*M* (13 mg/mL) magnesium chloride ($MgCl_2{\cdot}6H_2O$); glucose-6-phosphate dehydrogenase (40 U/mL) in 5 m*M* (1.5 mg/mL) sodium citrate ($C_6H_5O_7Na_3{\cdot}2H_2O$) (*see* **Note 1**).
4. Enzymes: for example, cDNA-expressed CYP4A11 (BD Biosciences, Discovery Labware, Woburn, MA) or human liver microsomes can be used. Dilute in assay buffer to give a working concentration of 2.5 mg of protein/mL and keep on ice (*see* **Note 2**).
5. Deproteinizing agent: 94% acetonitrile/6% glacial acetic acid.
6. Scintillation fluid: Ultima Flo M (Perkin-Elmer, Downers Grove, IL).

### 2.2. High-Performance Liquid Chromatography

1. Mobile phase A: 30% acetonitrile, 2 m*M* perchloric acid.
2. Mobile phase B: 100% methanol.
3. Nucleosil C18 column, 4.6 × 250 mm, 5-µm particle size (Sigma-Aldrich).
4. Radiometric-flow scintillation detector (Perkin-Elmer).

## 3. Methods

1. To each 1.5-mL microcentrifuge (incubation) tube, add 10 nmol of 5 mCi/mmol [1-$^{14}$C]lauric acid (100 µ*M* final concentration) (prepared as described in **Subheading 2.1., item 2**) (*see* **Note 3**).
2. Add the following to each incubation tube (total incubation volume of 100 µL):
   a. 64 µL of assay buffer.
   b. 5 µL of a solution containing 26 m*M* $NADP^+$, 66 m*M* D-glucose-6-phosphate, and 66 m*M* magnesium chloride.
   c. 1 µL of glucose-6-phosphate dehydrogenase (40 U/mL) in 5 m*M* sodium citrate.
3. Prewarm incubation tubes to 37°C, and add 20 µL of diluted enzymes (50 µg of protein) to initiate the enzymatic reaction (stagger each incubation with 15-s delay intervals) (*see* **Notes 4** and **5**).
4. Incubate the samples at 37°C for 15–30 min in a water bath (*see* **Note 6**).
5. Add 50 µL of ice-cold 94% acetonitrile/6% glacial acetic acid to stop the enzymatic reaction, and place the incubation tubes on ice.

6. Centrifuge the reaction mixture at 10,000*g* for 3 min.
7. Inject 100 µL of supernatant onto the HPLC column.
8. Run the column at 45–50°C (*see* **Note 7**) at a flow rate of 1 mL/min with a linear gradient from 70% mobile phase A, 25% mobile phase B to 53% phase A, 47% phase B over 22 min. Then changed the mobile phase to 100% phase B over 1 min and run the column for a further 9 min with 100% phase B. Under these conditons, the retention times are 20 min for 11-hydroxylauric acid, 21.5 min for 12-hydroxylauric acid, and 30 min for lauric acid (*see* **Note 8**).
9. Prepare blank incubation tubes by adding the complete incubation mixture but with heat-inactivated enzymes. Process the blank incubation tubes per **steps 4–8**.
10. To achieve quantification, use a flow-scintillation detector (Packard) with scintillation fluid at a flow rate of 3 mL/min with a 0.5-mL flow cell. Determine counting efficiency using a known quantity of $^{14}C$ with scintillation fluid and HPLC mobile phase in the same proportions as will be present during an analytical run (final volume of 20–50 mL) (*see* **Note 9**). Using the scintillation-fluid pump, fill the flow cell of the detector with this mixture. Measure the radioactivity (cycles per minute [cpm]). The counting efficiency is given by the cpm observed divided by the disintegration per minute (dpm) present in 0.5 mL (the volume of the flow cell). Activity is calculated according to the following equation:

$$\frac{[(\text{dpm metabolite}) \times (\text{total volume of incubation + stop addition})]}{[(\text{counting efficiency}) \times (2200\ \text{dpm/nCi}) \times (\text{specific activity in nCi/nmol}) \times (\text{mg protein}) \times \text{time} \times (\text{volume injected for analysis})]}$$

11. Calculate lauric acid 12-hydroxylation activity and express it as nanomoles of product formed/(minute·milligrams of microsomal protein) or as nanomoles of product formed/(minute·nanomoles of total P450).
12. Calculate net enzyme activity by subtracting the activity in the blank from each of the unknown samples (*see* **Note 10**).

## 4. Notes

1. Lauric acid 12-hydroxylation assays can also be carried out with nicotinamide adenine dinucleotide phosphate (NADPH) (e.g., 1 m*M* final concentration) in place of an NADPH-generating system (e.g., $NADP^+$, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase).
2. Prepare only sufficient amounts of dilute microsomes for each experiment. The remainder of the undiluted microsomes can be stored at –80°C for future use (*11*).
3. Reported apparent $K_m$ values for lauric acid 12-hydroxylation by human liver microsomes range from 13 to 49 µ*M* (*5,7*). The assay is conducted at a substrate concentration of 100 µ*M* in order to minimize the contribution of a higher-$K_m$ form (apparent $K_m$ approx 550 µ*M*) of lauric acid 12-hydroxylase in human liver microsomes (*7*).
4. With some P450 expression systems, it is necessary to reconstitute the recombinant P450 protein with NADPH–cytochrome P450 reductase, cytochrome-$b_5$, and lipid before initiating substrate oxidation (*4*). Conduct preliminary experiments to establish the amount of each of these required for optimal catalytic activity.
5. Conduct preliminary experiments to ensure that the assay is linear with respect to microsomal protein concentration.
6. Conduct preliminary experiments to ensure that the assay is linear with respect to incubation time.

7. Column temperature can range from room temperature to 50°C. The use of a controlled, elevated temperature provides greater reproducibility in retention times and lower column back-pressures.
8. These gradient and column conditions provide a baseline separation of 11-hydroxylauric acid and 12-hydroxylauric acid using a Waters Model 2690 Separations Module. Some adjustment in gradient conditions may be necessary for other analytical systems. The small difference in retention times between 11- and 12-hydroxylauric acid (approx 1.5 min) makes detection in an HPLC column efficient by fraction collection and scintillation counting impractical.
9. Counting efficiency should be determined in the presence of each of several different HPLC mobile-phase solvents because quenching may vary based on solvent composition. In our experience, the counting efficiency is constant at approx 0.84 across the mobile-phase gradient described in this application.
10. Lauric acid 12-hydroxylation activity in a panel of 17 human liver microsomes ranged from 0.3 to 1.9 nmol/(min·mg of microsomal protein) at a substrate concentration of 100 μ*M* (unpublished results).

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# 18

## An Isocratic High-Performance Liquid Chromatography Assay for CYP7A1-Catalyzed Cholesterol 7α-Hydroxylation

**David J. Waxman and Thomas K. H. Chang**

**Summary**

A normal-phase, isocratic high-performance liquid chromatography assay is described for cholesterol 7α-hydroxylation catalyzed by CYP7A1, which corresponds to the first and rate-limiting step in the conversion of cholesterol into bile acids. This method is based on the conversion of the primary cytochrome P450 metabolite, 7α-hydroxycholesterol, into 7α-hydroxy-4-cholesten-3-one in a reaction catalyzed by exogenous cholesterol oxidase, followed by chromatographic separation with monitoring at 254 nm. This technique is applicable to enzymatic studies for determination of cholesterol 7α-hydroxylation activity catalyzed by cDNA-expressed CYP7A1 and animal or human liver microsomes.

**Key Words:** Cytochrome P450; CYP7A1; cholesterol; 7α-hydroxycholesterol; 7α-hydroxy-4-cholesten-3-one; cholesterol oxidase; cholesterol 7α-hydroxylation activity.

## 1. Introduction

CYP7A1, the only member in the human CYP7A subfamily ***(1)***, catalyzes cholesterol 7α-hydroxylation, the first and rate-limiting step in the conversion of cholesterol into bile acids. CYP7A1 has been isolated from human liver and purifed to apparent homogeneity ***(2)***. Rodent model studies have established that CYP7A1 is highly regulated by physiological factors that influence hepatic bile acid biosynthesis, including cholesterol feeding, diurnal factors, and bile acids, which feedback inhibit the overall biosynthetic pathway in large part at the level of CYP7A1 gene expression ***(3)***. The regulation of CYP7A1 is complex and involves various nuclear orphan receptors, such as the liver X receptor α ***(4)*** and farnesoid X receptor ***(5)***. Relatively little is known about the factors that influence human CYP7A1 expression, although liver biopsy analyses have shown that hepatic CYP7A1 protein content is elevated in patients treated with the bile acid sequesterant cholestyramine ***(6)***. Purified CYP7A1 is catalytically active in cholesterol 7α-hydroxylation ***(2)*** and immunoinhibition experiments with anti-human CYP7A1 antibodies have shown that CYP7A1 accounts for most of

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the cholesterol 7α-hydroxylation activity in human liver microsomes ***(6)***. Thus, microsomal cholesterol 7α-hydroxylation activity can be used as a marker for human liver CYP7A1.

Several analytical methods have been developed to quantify hepatic microsomal cholesterol 7α-hydroxylation, including reverse-phase high-performance liquid chromatography (HPLC) ***(7)***, isotope dilution-mass spectrometry ***(8)***, and thin-layer chromatography ***(9)***. This chapter describes a normal-phase, isocratic HPLC assay for determination of cholesterol 7α-hydroxylation activity based on the conversion by cholesterol oxidase of the primary cytochrome P450 (P450) metabolite 7α-hydroxycholesterol into 7α-hydroxy-4-cholesten-3-one, which can be detected at 254 nm.

## 2. Materials

### *2.1. Assay*

1. Substrate: cholesterol (mol wt = 386.7) (Sigma-Aldrich, St. Louis, MO). Prepare a 1 m*M* (0.4 mg/mL) stock solution dissolved in toluene (*see* **Notes 1** and **2**). To prepare a 1 m*M* sonicated aqueous suspension of cholesterol, dry down an aliquot of the stock solution, add distilled water, and sonicate until a fine suspension is achieved.
2. Specialty reagents: 7α-hydroxycholesterol (Steraloids, Wilton, NH); cholesterol oxidase (Sigma-Aldrich). Reconstitute to give a stock solution of 12.5 U/mL in 10 m*M* potassium phosphate buffer, pH 7.4; 1 m*M* dithiothreitol;, and 20% (v/v) glycerol.
3. Metabolite standard: 7α-hydroxy-4-cholesten-3-one (enzymatically produced from 7α-hydroxycholesterol by cholesterol oxidase).
4. Assay buffer: 100 m*M* HEPES, pH 7.4, containing 0.025 m*M* ethylenediamine tetraacetic acid (EDTA), and 50 m*M* NaF.
5. Cofactor: prepare a 10 m*M* (8.3 mg/mL) stock solution of nicotinamide adenine dinucleotide phosphate (NADPH) and keep on ice (*see* **Notes 3** and **4**).
6. Enzymes: e.g., cDNA-expressed CYP7A1 or human liver microsomes. Dilute in assay buffer to a working concentration of 10 mg of protein/mL and keep on ice (*see* **Note 5**).
7. 5% (v/v) Sodium cholate.
8. Deproteinizing agent: methanol.
9. Extraction reagent: petroleum ether.

### *2.2. High-Performance Liquid Chromatography*

1. Mobile phase: hexane/2-propanol (80:20).
2. Alltech silica column, 4.6 × 250 mm, 5-μm particle size.
3. Ultraviolet (UV) detector at 254 nm.

## 3. Methods

1. Add the following to each assay tube (total incubation volume of 100 μL):
   a. 50 μL of assay buffer.
   b. 10 μL of 1 m*M* sonicated aqueous cholesterol suspension (100 μ*M* final concentration).
   c. 20 μL of P450-containing enzyme sample (to give 200 μg of protein; *see* **Notes 6** and **7**).
2. Prewarm the incubation tubes to 37°C and add 20 μL of 10 m*M* NADPH (1 m*M* final concentration) to initiate the enzymatic reaction (stagger each incubation with 15-s delay intervals).

3. Incubate the samples at 37°C for 20–30 min in a shaking water bath (*see* **Note 8**).
4. Add 150 μL of distilled water, 30 μL of 5% (v/v) sodium cholate, and 20 μL of cholesterol oxidase.
5. Incubate at 37°C for 10 min (*see* **Note 9**).
6. Add 300 μL of ice-cold methanol to stop the enzymatic reaction and then place the incubation tubes on ice.
7. Extract with 3 mL of petroleum ether.
8. Centrifuge the reaction mixture at 5000*g* for 5 min.
9. Transfer the extract to a clean test tube and evaporate under a gentle stream of nitrogen.
10. Reconstitute the residue with 100 μL of hexane/2-propanol (80:20).
11. Inject 20–40 μL/100 μL of reconstituted sample onto an HPLC column.
12. Run the column with a mobile phase of hexane/2-propanol (80:20) at a flow rate of 1 mL/min. Monitor the eluent at 254 nm. Under these conditions, the retention times are 4.8 min for cholesterol and 6.4 min for 7α-hydroxy-4-cholesten-3-one.
13. Measure the background activity in blank incubation tubes containing the complete incubation mixture but with heat-inactivated enzymes. Process the blank incubation tubes as per **steps 3–12**.
14. Prepare standards by adding a known amount of authentic 7α-hydroxycholesterol metabolite to tubes containing the complete incubation mixture but with heat-inactivated enzymes. Process the blank incubation tubes as per **steps 3–12**.
15. Determine the amount of product formation by comparison to a standard curve derived from 7α-hydroxy-4-cholesten-3-one.
16. Calculate cholesterol 7α-hydroxylation activity and express it as nanomoles of product formed/(minute·milligrams of microsomal protein) or as nanomoles of product formed/(minute·nanomoles of total P450) (*see* **Note 10**).

## 4. Notes

1. Cholesterol can also be added as a Tween-20 solution ***(9)*** or directly as low-density lipoprotein (LDL)-bound cholesterol (0.4 mg of cholesterol/mg of LDL).
2. Use a freshly prepared aqueous suspension of cholesterol for each experiment.
3. Prepare fresh solutions of NADPH for each experiment. Note that NADPH is light- and pH sensitive.
4. Cholesterol 7α-hydroxylation assays can also be carried out with an NADPH-generating system (e.g., $NADP^+$, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) in place of NADPH.
5. Prepare only sufficient amounts of dilute microsomes for each experiment. The remainder of the undiluted microsomes can be stored at –80°C for future use ***(10)***.
6. With some P450 protein expression systems, it is necessary to reconstitute the recombinant P450 protein with NADPH–cytochrome P450 reductase, cytochrome-$b_5$, and lipid prior to initiating substrate oxidation ***(11)***. Conduct preliminary experiments to establish the amount of each of these required for optimal catalytic activity.
7. Conduct preliminary experiments to ensure that the assay is linear with respect to microsomal protein concentration.
8. Conduct preliminary experiments to ensure that the assay is linear with respect to incubation time.
9. The enzymatically produced 7α-hydroxycholesterol metabolite is converted into the UV-active 7α-hydroxy-4-cholesten-3-one by cholesterol oxidase in order to increase the detection sensitivity of the assay ***(12)***. The latter standard is produced enzymatically by

incubating known amounts of 7α-hydroxycholesterol with cholesterol oxidase. For this method to be valid, it is essential to verify that the cholesterol oxidase effects a quantitative conversion of 7α-hydroxycholesterol into 7α-hydroxy-4-cholesterol-3-one.

10. A typical level of cholesterol 7α-hydroxylation activity in human liver microsomes is approx 0.02 nmol/(min·nmol of total P450) content (*2*).

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# 19

## Use of 7-Ethoxycoumarin to Monitor Multiple Enzymes in the Human CYP1, CYP2, and CYP3 Families

**David J. Waxman and Thomas K. H. Chang**

**Summary**

7-Ethoxycoumarin is metabolized by many cytochrome P450 enzymes active in foreign compound metabolism and has been used as a prototypic substrate to monitor P450 (P450) activity in both hepatic and extrahepatic tissues. A spectrofluorometric method is described for determination of P450-catalyzed 7-ethoxycoumarin *O*-deethylation. Following acidification of the incubation mixture, the enzymatic product, 7-hydroxycoumarin, is recovered by a double-extraction procedure and measured at an excitation wavelength of 370 nm and an emission wavelength of 450 nm. This method is applicable to enzymatic studies to determine the catalytic activity of cDNA-expressed human enzymes in the CYP1, CYP2, and CYP3 families, and 7-ethoxycoumarin *O*-deethylation activity in microsomes isolated from liver and other tissues.

**Key Words:** Cytochrome P450; CYP1; CYP2; CYP3; 7-ethoxycoumarin; 7-ethoxycoumarin *O*-deethylation; 7-hydroxycoumarin.

## 1. Introduction

Chapters 8–18 in this volume describe assay methods for determining enzyme activities that are frequently employed as markers for specific cytochrome P450 (P450) enzymes in human tissues. However, under certain circumstances, such as, when the objective is to verify that individual cDNA-expressed P450 enzymes are catalytically active, it is often advantageous to assay the panel of enzymes using a "general" P450 substrate, one that is metabolized by multiple P450 enzymes. An example of a general P450 substrate is 7-ethoxycoumarin. Enzyme kinetic analysis with human liver microsomes indicates that 7-ethoxycoumarin is metabolized by at least two P450 enzymes or by two groups of P450 enzymes that are kinetically distinguishable (*1*,*2*). The apparent $K_m$ and maximal specific activity (i.e., specific activity at saturating substate concentration) values for 7-ethoxycoumarin *O*-deethylation by human liver microsomes are 11–27 μ*M* and 0.05–0.21 nmol/(min·mg of microsomal protein), respectively, for the low-$K_m$ component. By contrast, for the high-$K_m$ component, the

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apparent $K_m$ is approx 150 μ*M* and the maximal specific activity is 0.35–0.95 nmol/(min·mg of microsomal protein) *(2)*. Experiments with recombinant human P450s have shown that multiple enzymes in the human CYP1, CYP2, and CYP3 families are active catalysts of 7-ethoxycoumarin *O*-deethylation *(3)*. Consequently, the 7-ethoxycoumarin *O*-deethylation assay has been used to verify the catalytic activity of a panel of recombinant human P450s in the CYP1, CYP2, and CYP3 families *(4–6)*.

This chapter describes a modification *(7)* of a widely used spectrofluorometric method *(8)* for the determination of 7-ethoxycoumarin *O*-deethylation activity. The formation of 7-hydroxycoumarin from 7-ethoxycoumarin can also be quantified by high-performance liquid chromatography with fluorescence detection *(9–11)*.

## 2. Materials

1. Substrate: 7-ethoxycoumarin (mol wt = 190.2) (Sigma-Aldrich, St. Louis, MO). Prepare a fresh 50 m*M* (9.5 mg/mL) stock solution in methanol (*see* **Notes 1** and **2**).
2. Metabolite standard: 7-hydroxycoumarin (umbelliferone; mol wt = 162.1) (Sigma-Aldrich).
3. Assay buffer: 100 m*M* potassium phosphate, pH 7.4, containing 20% (v/v) glycerol and 0.1 m*M* ethylenediamine tetra-acetic acid (EDTA) (*see* **Note 3**).
4. Cofactor: 10 m*M* (8.3 mg/mL) nicotinamide adenine dinucleotide phosphate (NADPH) stock solution. Prepare fresh and keep on ice (*see* **Notes 4** and **5**).
5. Enzymes: human recombinant P450 or human liver microsomes. Dilute in assay buffer to a working concentration of 1 or 2 mg of protein/mL and keep on ice (*see* **Note 6**).
6. Deproteinizing agent: 2 *M* HCl.
7. Extraction solvent: chloroform.
8. Back-extraction solution: 30 m*M* sodium borate ($Na_2B_4O_7$), pH 9.2.
9. Equipment including a spectrofluorometer and water bath.

## 3. Methods

1. Add the following to each incubation tube at room temperature (*see* **Note 3**) (total incubation volume of 200 μL):
   a. 156 μL of assay buffer.
   b. 4 μL of 50 m*M* 7-ethoxycoumarin (1 m*M* final concentration).
   c. 20 μL of diluted recombinant P450 (or human liver microsomes) (20 or 40 μg of protein) (*see* **Notes 7** and **8**).
2. Prewarm the incubation tubes to 37°C and add 20 μL of 10 m*M* NADPH (1 m*M* final concentration) to initiate the enzymatic reaction (stagger each incubation with 15-s delay intervals).
3. Incubate the samples at 37°C for 30 min in a shaking water bath (*see* **Note 9**).
4. Add 25 μL of ice-cold 2 *M* HCl to stop the enzymatic reaction and place the incubation tubes on ice.
5. Extract with 450 μL of chloroform (*see* **Note 10**).
6. Centrifuge the reaction mixture at 3000*g* for 5 min.
7. Transfer 300 μL of the organic phase (bottom layer), to a clean test tube and back-extract with 1 mL of 30 m*M* sodium borate, pH 9.2.
8. Repeat **step 6**.
9. Remove the aqueous (top) layer and measure the fluorescence at an excitation wavelength of 370 nm and an emission wavelength of 450 nm (*see* **Note 11**).

10. Prepare blank incubation tubes by adding the complete incubation mixture but with heat-inactivated recombinant P450 (or human liver microsomes). Process the blank incubation tubes per **steps 3–9**.
11. Prepare standards by adding a known amount (e.g., 0, 0.1, 0.2, 0.4, 0.8, and 1.2 nmol) of authentic 7-hydroxycoumarin metabolite to tubes containing the complete incubation mixture but with heat-inactivated recombinant P450 (or human liver microsomes). Process the incubation tubes containing the standards per **steps 3–9**.
12. Calculate the net fluorescence of each unknown sample and standard by subtracting the fluorescence reading of the blank from that of the unknown sample or the standard.
13. Plot a standard curve of net fluorescence vs the amount of authentic 7-hydroxycoumarin and determine the amount of product formation in each unknown sample by linear regression analysis.
14. Calculate 7-ethoxycoumarin *O*-deethylation activity and express it as nanomoles of product formed/(minute·milligrams of microsomal protein) or as nanomoles of product formed/(minute·nanomoles of total P450).

## 4. Notes

1. Use a freshly prepared stock solution of 7-ethoxycoumarin for each experiment.
2. Given that the activity of each P450 enzyme can be affected differentially by solvents such as methanol, ethanol, dimethyl sulfoxide, and acetonitrile ***(12)***, choose the appropriate solvent and concentration that will result in the least inhibition or stimulation of the cDNA-expressed P450 enzyme of interest.
3. Solubility considerations require that the assay tubes be prepared at room temperature, rather than on ice, and that glycerol be included in the incubation mixture in order to achieve true solubility for 1 m*M* 7-ethoxycoumarin. Alternatively, the final concentration of 7-ethoxycoumarin may be decreased to 0.2 or 0.5 m*M*, in which case the glycerol may be eliminated. Some P450s exhibit higher activity when assayed in the absence of glycerol.
4. Prepare fresh solutions of NADPH for each experiment. Note that NADPH is light- and pH sensitive.
5. The 7-ethoxycoumarin *O*-deethylation assay can also be carried out with an NADPH-generating system (e.g., $NADP^+$, D-glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) in place of NADPH.
6. Prepare only sufficient amounts of dilute microsomes for each experiment. The remainder of the undiluted microsomes can be stored at –80°C for future use ***(13)***.
7. With yeast and some other P450 protein expression systems, it is necessary to reconstitute the recombinant P450 protein with NADPH–cytochrome P450 reductase, cytochrome-$b_5$, and lipid (e.g., dilauroylphosphatidylcholine) before initiating substrate oxidation ***(5,14)***.
8. Conduct preliminary experiments to ensure that the assay is linear with respect to microsomal protein concentration.
9. Conduct preliminary experiments to ensure that the assay is linear with respect to incubation time.
10. "Wet" pipet tips with the organic solvent before use.
11. Determine the optimal excitation wavelength and emission wavelength to be used with each particular fluorimeter by examining the excitation and emission spectra of 7-ethoxycoumarin and 7-hydroxycoumarin ***(15)*** generated by that instrument.

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# 20

## Benzydamine *N*-Oxygenation as a Measure of Flavin-Containing Monooxygenase Activity

**Catherine K. Yeung and Allan E. Rettie**

**Summary**

Benzydamine is a nonsteroidal anti-inflammatory drug that undergoes flavin-containing monooxygenase (FMO)-dependent metabolism to a stable *N*-oxide. This metabolite can be quantified with high specificity and sensitivity by using a simple reverse-phase high-performance liquid chromatography (HPLC) assay with fluorescence detection. Studies with recombinant FMO enzymes demonstrate that FMO1 and FMO3 are the primary catalysts of benzydamine *N*-oxygenation, with minimal contributions from cytochrome P450 enzymes. Investigations conducted with human liver microsomes confirm that FMO3, in large part, is responsible for benzydamine *N*-oxide formation in this tissue. These features render benzydamine a useful in vitro probe for FMO activity in a wide range of tissues and cell types. In addition, benzydamine appears to be a suitable in vivo probe for human liver FMO3. This chapter provides a detailed account of the experimental protocol for determining rates of formation of benzydamine *N*-oxide by FMO-containing enzyme fractions.

**Key Words:** Flavin-containing monooxygenase; benzydamine; oxidation; catalysis.

### 1. Introduction

The flavin-containing monooxygenases (FMOs) are a family of microsomal enzymes that play a role in the oxidative metabolism of a broad range of substrates. Currently, five functional FMO isoforms have been characterized in humans, each designated by an Arabic number (FMO1–FMO5). FMO3 predominates in adult human liver tissue ***(1)***, with concentrations that can approach those of cytochrome P450 (P450) 3A4, the most abundant P450 ***(2)***. Both endogenous compounds, such as trimethylamine ***(3)***, and xenobiotics, including methimazole ***(4)***, cimetidine ***(5)***, clozapine ***(6)***, and benzydamine ***(7)***, undergo oxidation catalyzed by FMO isoforms. Of these substrates, benzydamine has been evaluated as a possible in vivo probe for phenotyping human FMO activity owing to its rapid and enzyme-selective *N*-oxygenation. This is primarily due to catalysis by human FMO isoforms 1 and 3, with minor involvement

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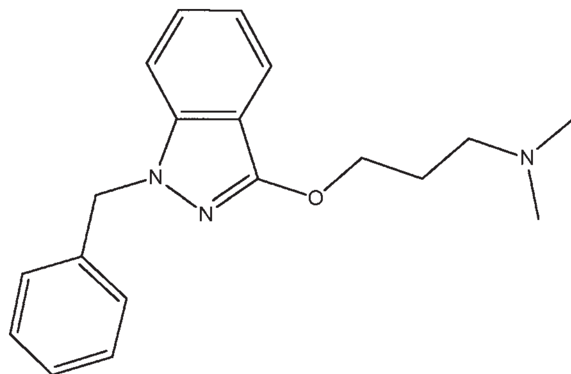


Fig. 1. Structure of benzydamine.

by FMO4, although extremely low levels of benzydamine *N*-oxide are also formed by human P450 isoforms CYP1A1, CYP1A2, CYP2C19, CYP2D6, and CYP3A4 ***(8)***. Studies with human tissue microsomes indicate that multiple organs contribute to benzydamine oxidation to varying degrees as follows: adult liver > kidney > fetal liver > intestine, in parallel with their FMO activities ***(3)***.

Benzydamine (**Fig. 1**), which acts pharmacologically by weakly inhibiting both prostaglandin G/H synthase (PGHS) 1 and 2 enzymes, exhibits anti-inflammatory, antipyretic, and antiedemal activities when administered orally or topically ***(9)***. An orally administered dose of 50 mg results in peak plasma concentrations of benzydamine *N*-oxide and the parent compound ranging from 0.2 to 0.6 μg/mL ***(10)***.

Analysis of benzydamine *N*-oxide formation has been utilized in systems as diverse as tissue slices ***(11)***, *in situ* microdialysis ***(12)***, and cryopreserved hepatocytes ***(13)***, as well as in conventional systems such as microsomal tissue incubations, isolated membrane preparations, and purified FMO from recombinant bacterial and insect cell expression systems. *Km* values for *N*-oxidation in recombinant FMO1, FMO3, FMO4, and FMO5 membranes from baculovirus-infected insect cells, at pH 7.4, were determined to be 60 ± 8 μ*M*, 80 ± 8 μ*M*, >3 m*M*, and >2 m*M*, respectively ***(8)***, and values in human liver microsomes ranged from 20 to 102 m*M* ($n$ = 35) ***(14)***. Maximal catalysis by FMO3 occurs at pH 9.0 ***(3)***, but enzyme incubations are often conducted at pH 7.4 in order to approximate physiological conditions. *N*-demethylation (to norbenzydamine) is catalyzed primarily by CYP3A4 but accounts for <10% of total benzydamine metabolism ***(13)***. Although benzydamine *N*-oxygenation is not FMO isoform specific, differential tissue distribution of FMO1 (kidney) and FMO3 (liver) allows the logical conclusion that hepatic FMO3 would be the predominant contributor to plasma levels of benzydamine *N*-oxide. Accordingly, orally administered benzydamine, with subsequent plasma monitoring of the *N*-oxide, would seem a suitable in vivo probe for human FMO3 ***(8,14)***, but its use has been restricted because it is available clinically only in some European countries.

# 2. Materials

## *2.1. Assay Components*

1. FMO-containing preparation (e.g., tissue microsomes, insect cell or bacterial membranes, purified FMO).
2. 0.1 *M* Potassium phosphate buffer, pH 7.4–9.0 (*see* **Note 1**). (Inspect stored solutions for cloudiness or precipitation, which may indicate bacterial contamination.)
3. Nicotinamide adenine dinucleotide phosphate (NADPH): 5 m*M* in 0.1 *M* phosphate buffer, pH 7.4–9.0 (*see* **Notes 2** and **3**).
4. Benzydamine (50 m*M* in methanol) (*see* **Note 4**).
5. Benzydamine *N*-oxide.
6. Dazidamine (0.1 mg/mL) in acetonitrile (internal standard) (*see* **Note 5**).
7. Shaking water bath equilibrated to 37°C.

## *2.2. High-Performance Liquid Chromatography*

1. Agilent Hypersil ODS C18 (5 mm/4.0 × 250 mm) column.
2. Mobile phase: 92% methanol/acetonitrile/water/29% $NH_4OH$ (50:40:10:0.05 [v/v/v/v]), 8% nanopure $H_2O$.
3. High-performance liquid chromatography with fluorescence detector.

# 3. Methods

The methods described outline assay of FMO activity using benzydamine as a substrate, and detection and quantification of the resultant metabolite, benzydamine *N*-oxide.

## *3.1. Incubation Conditions*

Typical FMO incubations require 10–300 pmol of heterologously expressed enzyme or 0.2–0.6 mg of microsomal protein.

1. In a total volume of 250 µL, combine the following:
   a. Enzyme (*see* **Notes 6** and **7**): typical FMO incubations require 10–300 pmol heterologously expressed enzyme or 0.2–0.6 mg of microsomal protein.
   b. NADPH (final concentration of 0.5 m*M*).
   c. Buffer sufficient to bring volume to 250 µL.
2. Mix thoroughly and incubate in a 37°C (*see* **Note 8**) shaking water bath (70 rpm) for 3 min (*see* **Note 9**).
3. Initiate the reaction with the addition of benzydamine substrate (typical final concentration of 1 m*M*), mix thoroughly, and return to the 37°C shaking water bath.
4. Incubate for 5–30 min with continued gentle agitation (70 rpm).
5. Quench the reaction with the addition of 250 µL (equal to incubation volume) of acetonitrile containing dazidamine (0.1 mg/mL) internal standard.
6. Place on ice for 5 min to allow full precipitation of proteins.
7. Pellet the proteins by centrifuging in a benchtop microcentrifuging at 14,000*g* for 5 min. The supernatant is suitable for immediate high-performance liquid chromatography (HPLC) analysis; exercise caution to avoid transferring any of the precipitated protein to the HPLC vial.

### 3.2. High-Performance Liquid Chromatography

1. Generate a standard curve for benzydamine *N*-oxide before each set of injections. Typically, combine 0, 1, 2, 5, or 10 pmol of benzydamine *N*-oxide with incubation buffer (and enzyme minus NADPH) to a total volume of 250 μL, followed by 250 μL of dazidamine internal standard (0.1 mg/mL in acetonitrile). Vortex the mixture and transfer to appropriate HPLC vials for analysis.
2. Analyze benzydamine, its *N*-oxide, and the internal standard using HPLC with fluorescence detection utilizing the following conditions:
   a. Agilent Hypersil column.
   b. Mobile phase: 92% methanol/acetonitrile/water/29% $NH_4OH$ (50:40:10:0.05 [v/v/v/v]), 8% nanopure $H_2O$, at a flow rate of 1.0 mL/min.
3. Monitor the effluent fluorometrically with an excitation wavelength of 307 nm and an emission wavelength of 377 nm. Approximate retention times are as follows, but elution times may vary significantly with different columns (*see* **Fig. 2**):
   a. Benzydamine *N*-oxide: 4 min.
   b. Dazidamine: 6 min.
   c. Benzydamine: 8 min.
   d. Norbenzydamine: 14 min (not detected in FMO assays).

## 4. Notes

1. Enzyme incubations are often performed at pH 7.4 in order to better approximate physiological conditions. However, FMO enzymes function maximally at higher pH, so expect lower rates of substrate oxidation when incubations are performed at nonoptimal pH.
2. Do not store the NADPH solution, and use only fresh NADPH. Oxidized NADPH develops a yellowish hue and should not be used. Keep the solution on ice once it has been reconstituted.
3. Alternatively, an NADPH-generating system consisting of 0.5 m*M* $NADP^+$, 6 m*M* glucose 6-phosphate, and 2.8 U/mL of glucose 6-phosphate dehydrogenase can be used.
4. Methanol and other solvents will inhibit enzyme activity at concentrations >1%. Stock substrate concentrations should be high enough such that the addition of methanolic substrate to enzymatic incubations results in a final methanol concentration of <1%.
5. Because this is not an extraction assay, the internal standard dazidamine is not strictly required. We use it mainly as a check on the proper functioning of the autoinjector.
6. For further information regarding the preparation of insect cell membranes, and flavin quantification via HPLC, *see* **ref. *2***.
7. FMOs are easily inactivated by exposure to high temperatures—heating microsomes at 50°C for 3 min will eradicate activity ***(15)***. Therefore, keep all enzyme-containing preparations on ice during manipulation. Additionally, multiple freeze/thaw cycles may cause protein degradation, so enzyme preparations should be stored in small aliquots.
8. Enzyme incubations involving mammalian FMOs should be performed at 37°C. However, incubations utilizing fish FMOs should be performed at a lower temperature (25°C).
9. For optimal metabolism by FMO enzymes, a 3-min preincubation period in the absence of substrate but presence of NADPH is recommended in order to form the catalytically active C4a-peroxyflavin species ***(16)***.

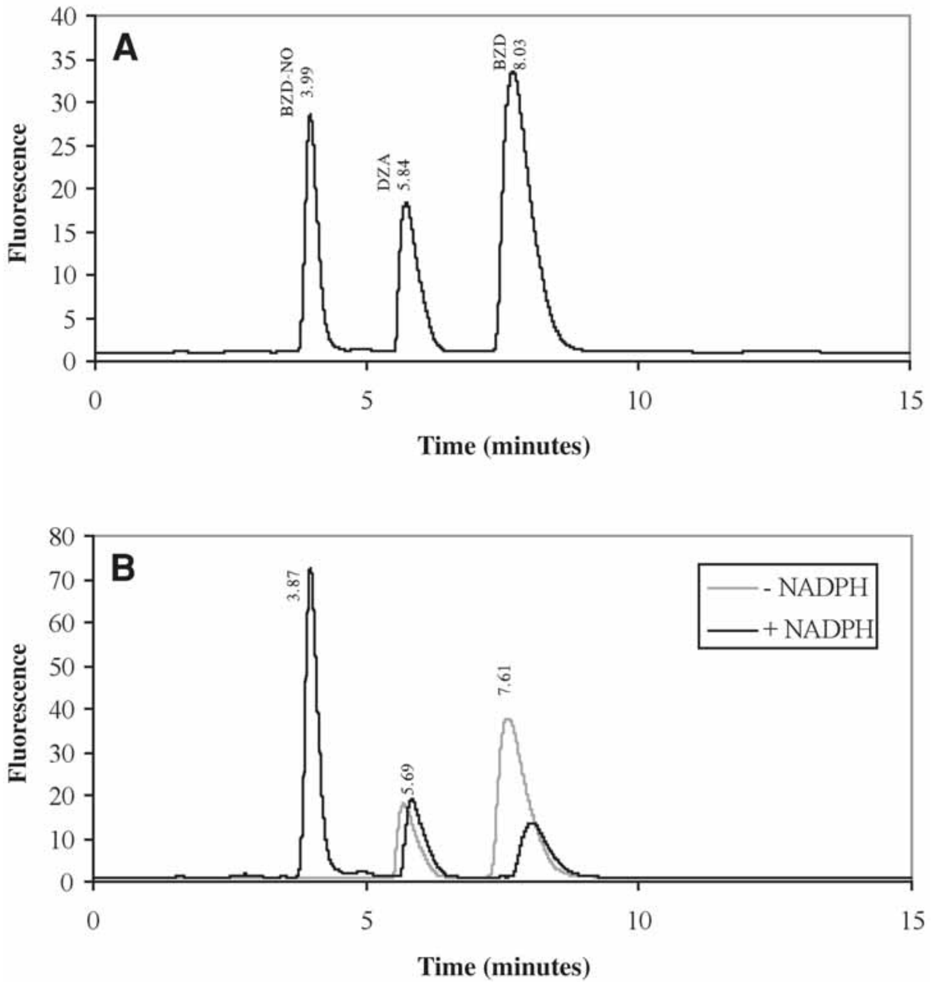


Fig. 2. Benzydamine assay sample chromatographs. **(A)** Benzydamine standards: BZD, benzydamine; BZD-NO, benzydamine *N*-oxide; DZA, dazidamine. **(B)** FMO3 incubations with benzydamine in presence and absence of nicotinamide adenine dinucleotide phosphate (NADPH).

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# Prochiral Sulfoxidation as a Probe for Flavin-Containing Monooxygenases

**Catherine K. Yeung and Allan E. Rettie**

**Summary**

Asymmetric aryl alkyl sulfides (R-S-R') are metabolized by flavin-containing monooxygenase (FMO) and cytochrome P450 enzymes to enantiomerically enriched sulfoxide products (R-SO-R') that are readily analyzed with a host of commercially available chiral stationary phases. Prochiral sulfoxidation of probe compounds based on *p*-tolyl methyl sulfide is a particularly useful method for discriminating among FMO1, FMO3, and FMO5, because the stereochemistry of the resulting products is isoform dependent, but apparently species independent. If studies are performed with crude tissue microsomal preparations, the cytochrome P450 component must be quenched to unmask catalysis specifically by the FMO component of the tissue. This chapter details experimental protocols for stereochemical analysis of sulfoxides generated from methyl, ethyl, *n*-propyl, and *n*-butyl *p*-tolyl sulfide by purified FMO isoforms and tissue microsomal preparations.

**Key Words:** Flavin-containing monooxygenase; arylalkyl sulfide; sulfoxidation; oxidation.

## 1. Introduction

The flavin-containing monooxygenases (FMOs) are a family of microsomal nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen-dependent enzymes that catalyze the oxygenation of nucleophilic nitrogen, sulfur, phosphorus, and selenium compounds) ***(1,2)***. To date, five catalytically active isoforms have been characterized and designated by an Arabic numeral (FMO1–5) ***(2)***. A sixth, truncated, putatively nonfunctional isoform has also been identified likely is physiologically insignificant ***(3)***. The FMOs share 50–55% amino acid sequence identity and have approx 87% identity among species orthologs ***(4)***. The catalytic mechanism has been studied in detail for only pig FMO1, but all isoforms are thought to share a common mechanism of action. The catalytic cycle involves the sequential binding to the enzyme of NADPH and oxygen to generate a reactive flavin adenine dinucleotide (FAD) C-4a

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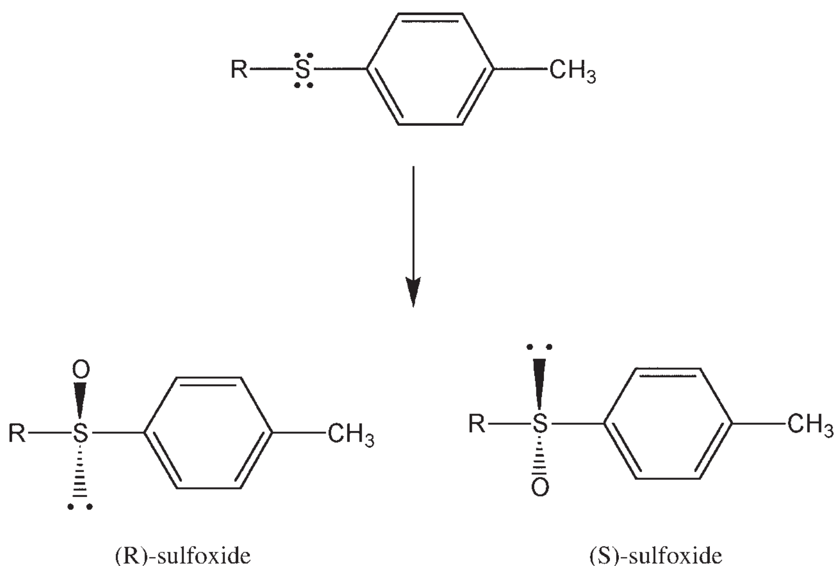


Fig. 1. Structure and metabolic fate of arylalkyl sulfides.

hydroperoxide *(5)*. Substrate nucleophiles attack the distal oxygen of the hydroperoxide with subsequent transfer of oxygen to the substrate and resultant formation of a hydroxyflavin species. The rate-limiting step in FMO catalysis is the subsequent decomposition of the hydroxyflavin, or $NADP^+$ release, as opposed to substrate binding or oxygen transfer *(6)*. This provides a rational explanation for the experimental observation that the $V_{max}$ for most FMO-dependent reactions is relatively constant for a wide variety of substrates *(5)*. Therefore, differentiating the various isoforms via kinetic parameters is generally limited to differences in substrate *Km*, and hence, other techniques are desirable.

In the early 1980s, Walsh et al. *(7)* examined the stereoselective sulfoxidation of ethyl *p*-tolyl sulfide by porcine liver FMO (FMO1) and observed formation of the (*R*) enantiomer of ethyl *p*-tolyl sulfoxide in 90% enantiomeric excess (*see* **Fig. 1**, where *R* = ethyl). This prompted us to develop a homologous series of arylalkyl sulfides (methyl, ethyl, propyl, butyl, and so on) as potential stereochemical probes for FMO *(8)*. These compounds were selected, in part, because of their facile synthesis as well as that of the enantiomerically pure sulfoxide standards. The latter are obtained conveniently via reaction of commercially available, diastereomerically pure (+) and (–) *p*-tolyl methylsulfinate with the appropriate alkyl Grignard reagent *(9)*. Data from several studies have demonstrated that the stereochemical composition of *n*-alkyl sulfoxide metabolites generated by various FMO isoforms is isozyme dependent *(7,10–12)* and also appears to be species *independent* *(10,13,14)*, although the latter has not been tested exhaustively. Methyl *p*-tolyl sulfide is recommended as the primary substrate because *(1)* both the substrate and the respective (*R*)- and (*S*)-sulfoxide metabolites are commer-

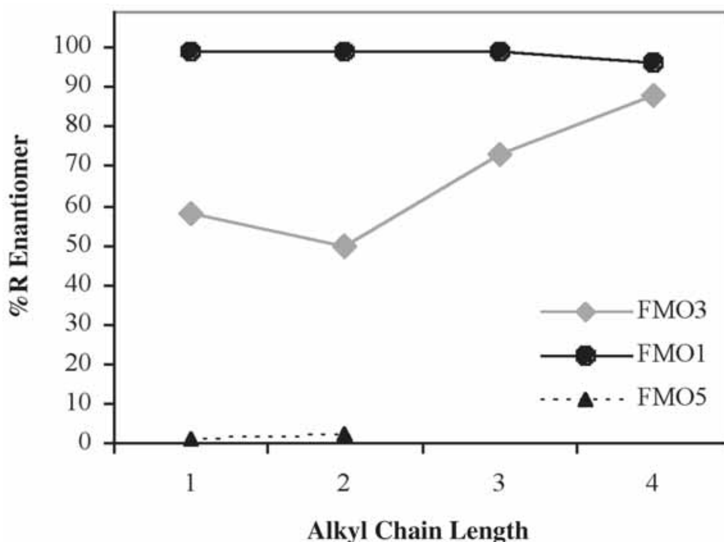


Fig. 2. Arylalkyl sulfoxidation profile for human FMO isoforms 1, 3, and 5.

cially available, and *(2)* because this substrate alone can distinguish among FMO1, FMO3, and FMO5 catalysis based on the stereochemistry of the sulfoxide products (**Fig. 2**). The differential processing of prochiral substrates by FMO may reflect variations in substrate-binding energetics contained within the enzyme-active site *(8)*.

This chapter details experimental protocols for stereochemical analysis of sulfoxides generated from methyl, ethyl, *n*-propyl, and *n*-butyl *p*-tolyl sulfide by both tissue microsomal preparations, which may contain cytochrome P450 (P450) enzymes (*see* **Subheading 3.1.**), and a more rapid protocol for use with purified or heterologously expressed FMO isoforms (*see* **Subheading 3.2.**).

## 2. Materials

### 2.1. FMO-Catalyzed Chiral Sulfoxidation in Microsomal Samples Containing P450 Enzymes

#### 2.1.1. Assay Components

1. FMO-containing preparation (e.g., human liver microsomes).
2. Glycine (100 m*M*) and potassium pyrophosphate (25 m*M*) buffer, pH 8.5.
3. 2% (v/v) Lubrol aqueous solution.
4. Methyl *p*-tolyl substrate (100 m*M* stock solution in methanol) (*see* **Note 1**).
5. Methyl *p*-tolyl sulfoxides, (*R*) and (*S*), as high-performance liquid chromatography (HPLC) standards (0.1 mg/mL of aqueous solution).
6. Ethyl *p*-tolyl sulfoxide (0.05 mg/mL of aqueous solution)—internal standard.
7. NADPH: 5 m*M* in glycine-pyrophosphate buffer (*see* **Note 2**).
8. Dichloromethane.

9. Shaking water bath equilibrated to 37°C.
10. Glass tubes, 16 × 125 mm (some of which should be silanized to aid recovery of metabolite) or scintillation vials.

#### 2.1.2. High-Performance Liquid Chromatography

1. Silica HPLC column (Whatman Partisil 10 PAC, 4.6 × 250 mm).
2. Chiral HPLC column (Regis [R,R]-Whelk-O 1, 4.6 × 250 mm).
3. Mobile phase:
   a. HPLC-grade hexane and isopropyl alcohol (95:5 [v/v]).
   b. Mobile phase (HPLC-grade hexane and isopropyl alcohol [60:40 (v/v)]).
   c. HPLC with an ultraviolet (UV) detector.

### 2.2. FMO-Mediated Stereoselective Sulfoxidation by Heterologously Expressed or Purified FMOs

#### 2.2.1. Assay Components

1. FMO-containing preparation (e.g., insect cell or bacterial membranes, purified FMO).
2. 0.1 *M* Potassium phosphate buffer, pH 7.4–9.0 (*see* **Note 3**).
3. Alkyl *p*-tolyl substrate (100 m*M* stock solution in methanol) (*see* **Note 1**).
4. Alkyl *p*-tolyl sulfoxides, (*R*) and (*S*), as HPLC standards (0.1 mg/mL of aqueous solution).
5. NADPH (5 m*M* in 0.1 *M* potassium phosphate buffer) (*see* **Note 4**).
6. Perchloric acid (50% aqueous solution).
7. Shaking water bath equilibrated to 37°C.

#### 2.2.2. High-Performance Liquid Chromatography

1. Chiral HPLC column (Regis [R,R]-Whelk-O 1, 4.6 × 250 mm).
2. Mobile phase (HPLC-grade acetonitrile + 0.5% acetic acid in nanopure water, pH 4.7, in a 25:75 ratio).
3. HPLC with a UV detector.

## 3. Methods

### 3.1. FMO-Catalyzed Chiral Sulfoxidation in Microsomal Samples Containing P450 Enzymes

Incubations with tissue microsomes (e.g., liver, intestine, kidney) that contain endogenous P450 require additional treatment in order to minimize the contribution of these enzymes to sulfoxide formation. This can be accomplished by adding a P450 inhibitor (*N*-benzylimidazole; 1 m*M*) to the incubation ***(15)***. Alternatively, a detergent such as Lubrol can be added to inactivate P450 while maintaining FMO activity. Details of the latter approach, which requires an extraction assay, are briefly summarized here. Further information is detailed in **ref. *10***. Determination of kinetic parameters requires the addition of an internal standard before extraction in order to account for variable extraction efficiency.

The methods described outline incubation of FMO using methyl *p*-tolyl sulfide as a substrate; and detection and quantification of the resultant metabolites, (*R*) and (*S*) methyl *p*-tolyl sulfoxides, in complex tissue microsomal samples, which contain P450 enzymes.

### *3.1.1. Incubation Conditions*

1. In a total volume of 2.97 mL, combine the following:
   a. Enzyme (*see* **Note 5**): typical incubations contain 1–3 mg of microsomal protein.
   b. NADPH (0.5 m*M* final concentration) (*see* **Note 2**).
   c. Detergent: Lubrol to a 0.2% final concentration (*see* **Note 6**).
   d. Buffer: glycine-pyrophosphate, pH 8.5 (*see* **Note 6**), to bring the volume to 2.97 mL.
2. Mix thoroughly in a 16 × 125 mm-glass test tube, and incubate in a 37°C (*see* **Note 7**) shaking water bath (70 rpm) for 3 min (*see* **Note 8**).
3. Initiate the reaction with the addition of 30 μL of substrate (final substrate concentration of 1 m*M*), mix thoroughly, and return to the water bath. Incubate for 5–30 min with continued gentle agitation (70 rpm).
4. Quench the reaction with the addition of 7 mL of ice-cold dichloromethane. Add 100 μL (5 μg) of internal standard solution, followed by approx 0.5 g of NaCl, and vortex gently for 1 min.
5. Centrifuge the samples at low speed (500*g* for 4 min) to facilitate separation of the organic and aqueous phases. Aspirate the aqueous (upper) layer and discard.
6. Transfer the organic (lower) layer, avoiding the NaCl, to a clean tube and evaporate under a stream of nitrogen.
7. Add 50 μL of isopropanol to dissolve the dried sample being careful to wash down the sides of the tube to dissolve material that may be adhered. This solution is suitable for injection onto a silica HPLC column for separation and collection of the sulfoxide metabolite.

### *3.1.2. High-Performance Liquid Chromatography*

This protocol requires *two* normal-phase chromatographic steps. If product quantification is desired, a standard curve for methyl *p*-tolyl sulfoxide should be generated before each set of incubations. A typical standard curve is generated by spiking metabolic incubation components (minus NADPH) with racemic methyl sulfoxide (0–100 nmol) and 5.0 μg of ethyl *p*-tolyl sulfoxide, and then following the extraction steps outlined in **Subheading 3.1.1.** A linear relationship will be observed between the quantity of methyl *p*-tolyl sulfoxide added and the peak area ratio of methyl *p*-tolyl sulfoxide:ethyl *p*-tolyl sulfoxide.

#### 3.1.2.1. Step 1 of Protocol

The first chromatographic step requires a silica HPLC column a mobile phase of hexane/isopropyl alcohol (95:5 [v/v]), and a flow rate of 2.0 mL/min.

1. Inject prepared sample. Monitor the effluent at UV $\lambda_{absorbance}$ = 254 nm. Approximate retention times are listed in **Table 1,** but retention times may vary based on flow rate, degree of column condition, and buffer composition.
2. Collect the methyl sulfoxide peak in a clean, silanized glass tube and evaporate under a stream of nitrogen.

#### 3.1.2.2. Step 2 of Protocol

The second chromatographic step requires a chiral column (*see* **Note 9**), a mobile phase of hexane/isopropyl alcohol (60:40 [v/v]), and a flow rate of 1.0 mL/min.

**Table 1**
**Normal-Phase HPLC Retention Times of Alkyl *p*-Tolyl Sulfoxides**

| Alkyl chain length | Sulfoxide retention time (min)[a] | (R)-Sulfoxide retention time (min)[b] | (S)-Sulfoxide retention time (min)[b] |
|---|---|---|---|
| Methyl | 14.5 | 11.2 | 13.5 |
| Ethyl | 8.6 | 9.5 | 11.5 |
| Propyl | 5.3 | 8.3 | 10.7 |
| Butyl | 4.1 | 7.8 | 10.2 |

[a]Approximate retention time in minutes of alkyl *p*-tolyl sulfoxide using a nonchiral Whatman Partisil 10 PAC 4.6 × 250 mm column with a mobile phase consisting of 95% hexane, 5% isopropyl alcohol at a flow rate of 1.0 mL/min.

[b]Approximate retention times in minutes of pure enantiomers of alkyl *p*-tolyl sulfoxides using a chiral Regis (R,R)-Whelk-O 1, 4.6 × 250 mm column with a mobile phase consisting of 60% hexane, 40% isopropyl alcohol at a flow rate of 1.0 mL/min.

1. Reconstitute the sample (*see* **step 2** in **Subheading 3.1.2.1.**) in 50 µL of mobile phase before injection.
2. Monitor the effluent at UV $\lambda_{absorbance}$ = 254 nm. Approximate retention times are provided in **Table 1**.

### *3.2. FMO-Mediated Stereoselective Sulfoxidation by Heterologously Expressed or Purified FMOs*

This rapid, quantitative assay is useful primarily for determining enzyme kinetics and product stereochemistry from heterologously expressed or purified FMO preparations (*see* **Note 10**).

The methods described outline incubation of FMO using alkyl *p*-tolyl sulfide as a substrate, and detection and quantification of the resultant (*R*) and (*S*) alkyl *p*-tolyl sulfoxides.

#### *3.2.1. Incubation of Enzyme*

1. In a total volume of 495 µL, combine the following:
   a. Enzyme: Typical incubations require 20–200 pmol of heterologously expressed FMO.
   b. NADPH (final concentration of 0.5 m*M*) (*see* **Note 2**).
   c. Buffer: 0.1 *M* potassium phosphate buffer, pH 7.4–9.0 (*see* **Note 3**), to bring the total volume to 495 µL.
2. Mix thoroughly in a 1.8-mL microcentrifuge tube and incubate at 37°C in a shaking water bath (70 rpm) for 3 min (*see* **Note 8**).
3. Initiate the reaction with the addition of 5 µL of substrate (final substrate concentration of 1 m*M*), mix thoroughly, and return to the water bath. Incubate for 5–30 min with continued gentle agitation (70 rpm).
4. Quench the reaction with the addition of 5 µL of perchloric acid (final concentration of 1%), and place on ice for 5 min to allow full protein precipitation. Pellet the protein by

centrifuging in a benchtop microcentrifuge (14,000*g* for 10 min). The supernatant is suitable for immediate HPLC analysis and should be transferred to appropriate HPLC vials. Exercise caution to avoid transferring any of the insoluble protein, which may obstruct the HPLC column.

### *3.2.2. High-Performance Liquid Chromatography*

If product quantification is desired, a standard curve for the alkyl *p*-tolyl sulfoxide should be generated before each set of incubations. A typical standard curve is generated by spiking metabolic incubations (minus NADPH) with a range of sulfoxide concentrations, then acidifying and centrifuging the sample before injection on the HPLC column, as detailed in **Subheading 3.2.1.** UV absorbance at 254 nm has been shown to be linear within a range from 10 to at least 300 pmol injected on a column. If only a measure of the *R*:*S* sulfoxide ratio is desired, this can be obtained directly from comparison of the peak areas for the sulfoxide enantiomers.

The alkyl *p*-tolyl sulfoxides can be analyzed using HPLC (**Fig. 3**) with UV detection utilizing the following conditions:

1. Chiral column, for example, (R,R)-Whelk-O 1.
2. Mobile phase: solvent ratios will vary depending on the alkyl chain length, HPLC system, and degree of column conditioning, but approximate guidelines are given in **Table 2**.

The effluent is monitored at UV $\lambda_{absorbance}$ = 254 nm. Approximate retention times are provided in **Table 2**. Sample chromatographs are displayed in **Fig. 3**.

## 4. Notes

1. Methanol and other solvents will inhibit enzyme activity at concentrations >1%. Stock substrate concentrations should be high enough such that the addition of methanolic substrate to enzymatic incubations results in a final methanol concentration of <1%.
2. Alternatively, an NADPH-generating system consisting of 0.5 m*M* $NADP^+$, 6 m*M* glucose 6-phosphate, and 2.8 U/mL of glucose 6-phosphate dehydrogenase can be used.
3. Enzyme incubations are often performed at pH 7.4 in order to simulate physiological conditions. However, FMO enzymes function maximally at higher pH (8.5–9.0), so expect lower rates of substrate oxidation near physiological pH.
4. Do not store the NADPH solution, and use only fresh NADPH. Oxidized NADPH develops a yellowish cast and should not be used. Keep the solution on ice once it has been reconstituted.
5. FMOs are easily inactivated by exposure to high temperature—heating microsomes at 50°C for 3 min will destroy activity ***(15)***. Therefore, keep all enzyme-containing preparations on ice during manipulation. Additionally, multiple freeze/thaw cycles may cause protein degradation, hence, aliquoting of protein samples is highly recommended.
6. Conducting the incubation at pH 8.5, together with detergent treatment, helps minimize P450-mediated metabolism.
7. Enzyme incubations involving mammalian FMOs should be performed at 37°C. However, incubations utilizing nonmammalian fish FMOs should be performed at a lower temperature (25°C).
8. For optimal metabolism by FMO enzymes, a 3-min preincubation period in the absence of substrate but the presence of NADPH is recommended, in order to form the catalytically active C4a-peroxyflavin species ***(6)***.

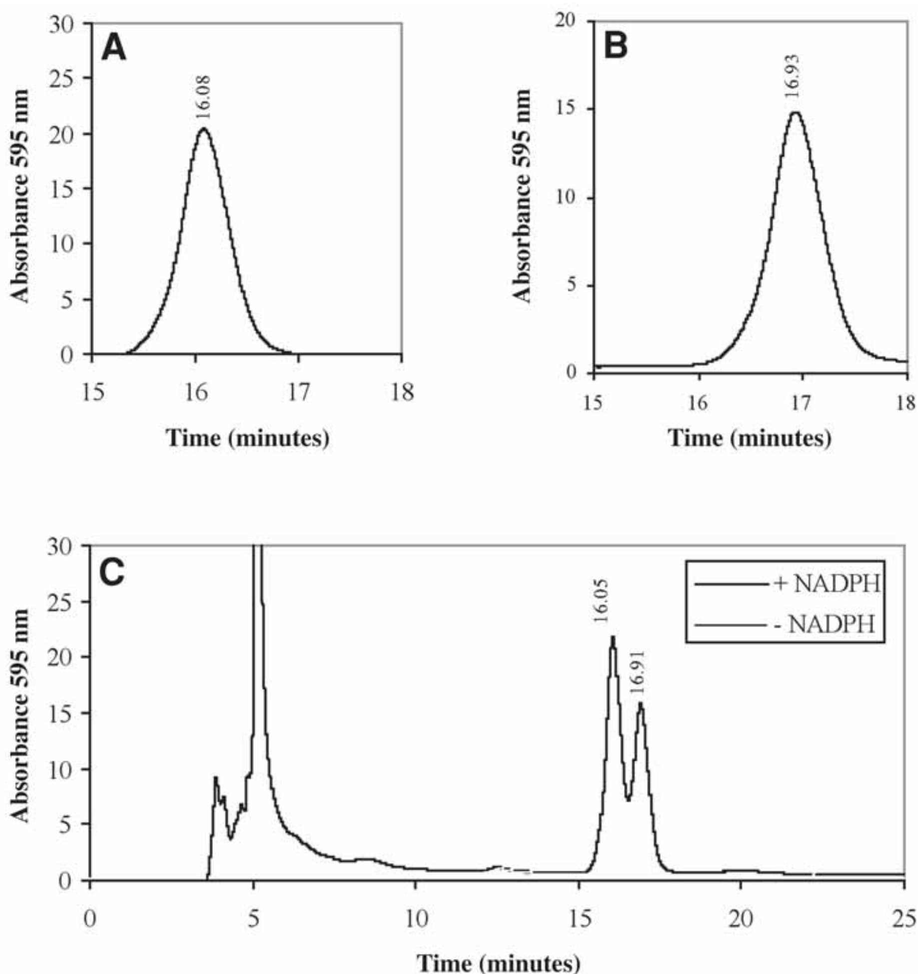


Fig. 3. Sample chromatographs of **(A)** (*R*)- and **(B)** (*S*)-methyl *p*-tolyl sulfoxide standards and **(C)** FMO3 incubations in the presence and absence of nicotinamide adenine dinucleotide phosphate (NADPH).

9. The Regis -Whelk-O 1 column is convenient because it can be used in either normal or reverse-phase mode. However, resolution of the sulfoxide metabolites is not usually complete. If better resolution is required, the use of a Daicel Chiralcel OB column with a hexane/isopropyl alcohol mobile phase is recommended ***(10)***.
10. For further information regarding the preparation of insect cell membranes and flavin quantification via HPLC, *see* **ref. *16***.

**Table 2**
**Approximate Reverse-Phase HPLC Retention Times of Alkyl *p*-Tolyl Sulfoxides Using a Regis (R,R)-Whelk-O 1, 4.6 × 250 mm Column With a Mobile Phase of Acetonitrile + 0.5% Acetic Acid, pH 4.7 (25:75), at a Flow Rate of 0.7 mL/min**

| Alkyl chain length | (*R*) retention time (min) | (*S*) retention time (min) |
|---|---|---|
| Methyl | 16 | 17 |
| Ethyl | 25 | 27 |
| Propyl | 40 | 44 |
| Butyl | 69 | 75 |

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# 22

# Targeting Antipeptide Antibodies Toward Cytochrome P450 Enzymes

**Robert J. Edwards**

**Summary**

An approach to raising antibodies is described that can be applied to the majority of cytochrome P450 (P450) enzymes. Its application is limited only by the availability of suitable protein sequence information. The method is based on immunizing animals with synthetic peptides that mimic small regions of target proteins. In practice, this has proved to be a relatively simple, rapid, and effective method of producing antibodies. The antibodies are particularly suited to immunoblotting (Western blotting) and immunocytochemistry. Unlike antibodies produced by other techniques, the epitopes of these antibodies are predetermined, allowing them to be directed toward specific regions of P450. Detailed practical information is described on the selection of regions to target, the synthesis and conjugation of peptides to carrier protein, immunization, and the assessment of the resultant antisera. The approach is illustrated using examples of antibodies targeted against rat and human P450.

**Key Words:** Antipeptide antibodies; cytochrome P450; synthetic peptides; immunization; conjugation; human; rat; immunoblotting; Western blotting.

## 1. Introduction

Antibodies against cytochrome P450 (P450) enzymes can be produced by immunizing animals with synthetic peptides that mimic small regions of the apoprotein. Because the amino acid sequences of a large number of P450 enzymes are known ***(1)***, this approach to raising antibodies can be applied to the majority of P450 enzymes in human, rat, mouse, and other species. This is a relatively simple, rapid, and effective method of producing antibodies ***(2)***.

The antipeptide approach to antibody production overcomes the need to use purified P450 enzymes as immunogens. Purification of P450 can be an arduous procedure and is especially difficult in tissues that have a low P450 content, although the use of heterologous expression systems can overcome some of the problems ***(3)***. Nevertheless, both polyclonal and monoclonal antibodies raised against purified P450 may

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crossreact with other P450 enzymes ***(4–6)***. By contrast, a high degree of binding specificity can be achieved by directing antipeptide antibodies toward unique regions of the target antigen. Successfully produced antipeptide antibodies recognize both native and denatured P450; hence, these antibodies are particularly suited to immunoblotting ***(7,8)*** and immunocytochemistry using formalin-fixed sections ***(9,10)***. Unlike antibodies produced by other techniques, the epitope of an antipeptide antibody is predetermined; thus, antibodies can be directed toward specific surface regions of P450. The structure and function of P450 enzymes can be studied using such antibodies through immunoinhibition of enzyme activity ***(11,12)***.

In this chapter, methods for the production of antibodies suitable for immunoblotting studies are described.

## 2. Materials

1. Lyophilized peptide (*see* **Notes 1–8**).
2. Keyhole limpet hemocyanin (KLH): this may be purchased as a partially purified product in 65% saturated ammonium sulfate (Calbiochem, Nottingham, UK). Dissolve 250 mg of KLH in 20 mL of water and dialyze five times against 1 L of 0.1 *M* sodium phosphate buffer, pH 7.2. Measure the protein concentration of the solution and store frozen at –20°C in 5-mL aliquots.
3. 0.05 *M* sodium phosphate buffer, pH 6.0.
4. Freshly prepared 0.01 *M* 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) dissolved in 0.1 *M* potassium phosphate buffer, 1 m*M* ethylene diamine tetra-acetic acid (EDTA), pH 8.0.
5. 2-Mercaptoethanol.
6. Freshly prepared 0.15 *M* *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) dissolved in dimethylformamide (DMF).
7. 1 *M* NaOH.
8. Sephadex G-25 column (25 × 2.2 cm) equilibrated in 0.05 *M* sodium phosphate buffer, pH 6.0.
9. Phosphate-buffered saline (PBS): 1.5 m*M* $KH_2PO_4$, 8.1 m*M* $Na_2HPO_4$, 2.7 m*M* KCl, 137 m*M* NaCl, pH 7.5.
10. Freund's complete and Freund's incomplete adjuvants (stable at 4°C for >1 yr).

## 3. Methods

### *3.1. Conjugation of Peptide to Carrier Protein*

1. Weigh 10 µmol of thiopeptide (*see* **Notes 1–8**) and dissolve in 2 mL of 0.05 *M* sodium phosphate buffer, pH 6.0 (*see* **Note 9**).
2. Remove 25 µL and dilute to 2.5 mL in 0.1 *M* potassium phosphate buffer, 1 m*M* EDTA, pH 8.0. Prepare a series of standard solutions between 0 and 100 µ*M* 2-mercaptoethanol in 0.1 *M* potassium phosphate buffer, 1 m*M* EDTA, pH 8.0. To 1-mL aliquots of diluted thiopeptide and standard solutions of 2-mercaptoethanol, add 0.05 mL of DTNB solution and mix. The presence of thiol is indicated by production of a yellow solution. Measure the absorbance of samples at 412 nm, and quantify the amount of thiopeptide present by comparing with the standards.
3. In preparation for conjugation to the thiopeptide, derivatize KLH with MBS (*see* **Notes 10** and **11**). Typically, 20 mg of KLH dissolved in 5 mL of 0.1 *M* sodium phosphate buffer, pH 7.2, is used. While mixing, add over a period of 3–5 min 95 µL of MBS solu-

tion (i.e., 0.22 mg of MBS/mg of KLH), and allow to react for 30 min.

4. Purify derivatized KLH by gel filtration using a Sephadex G-25 column equilibrated in 0.05 *M* sodium phosphate buffer, pH 6.0, at a flow rate of 2.5 mL/min. Monitor the absorbance of the eluent at 280 nm and collect 5-mL fractions. MBS-derivatized KLH is eluted over 10–15 mL in the void volume of the column.
5. Mix thiopeptide and MBS-derivatized KLH at a ratio of 1 µmol of thiopeptide to 2.4 mg of KLH. Adjust the mixture to pH 7.5 using 1 *M* NaOH. Mix for 2 h at room temperature and then dialyze three times against 500 mL of PBS (*see* **Notes 12** and **13**).

### 3.2. Immunization (see Notes 14 and 15)

1. Collect a preimmune blood sample from a marginal ear vein of a 3-kg male New Zealand White rabbit.
2. Allow the blood to clot and centrifuge at 950*g* for 15 min. Collect the serum and store at –20°C.
3. Dilute KLH-peptide conjugate to 0.4 mL in PBS.
4. Place 0.75 mL of Freund's complete adjuvant in a 5-mL vial, and while vortex mixing, gradually add 0.75 mL of KLH-peptide conjugate solution. This should produce a thick emulsion.
5. Inject 1 mL of the mixture of Freund's adjuvant and peptide conjugate (200 µg) into the rabbit subcutaneously at four sites (50 µg/site). All subsequent injections are prepared as described in **step 4**, except that Freund's incomplete adjuvant is used.
6. After 2 wk, inject 200 µg of peptide conjugate in Freund's adjuvant subcutaneously at four sites (50 µg/site).
7. After a further 2 wk, administer 200 µg of the peptide conjugate in Freund's adjuvant intramuscularly at two sites (100 µg/site).
8. One week later, collect a blood sample from a marginal ear vein and prepare the antiserum as described in **step 2**.
9. Test the antiserum for binding to P450 enzymes (*see* **Notes 16–18**).
10. Administer booster injections intramuscularly at approx 1-mo intervals, and collect blood 1 wk later on each occasion.

## 4. Notes

1. Up-to-date information on P450 sequences can be found on David Nelson's Cytochrome P450 Homepage at http://drnelson.utmem.edu/CytochromeP450.html and in the Directory of P450-Containing Systems, which can be found at www.icgeb.trieste.it/p450/.
2. A number of factors need to be considered when selecting a peptide for immunization. If an antibody is required for immunoblotting and/or immunocytochemistry, then the choice can be quite straightforward. Antibodies directed toward the C-terminus of P450 are the simplest to produce and the most reliable for reacting with the target P450 enzyme *(2)*. The reason is that by coupling through its N-terminus, the orientation of the peptide is the same as the C-terminus of the P450 enzyme and the resultant antibodies recognize both equally well. Antibodies that bind to most of the major forms of P450 in humans and rats have been produced in this way (several examples are shown in **Figs. 1** and **2**). Alternatively, antibodies may be directed toward internal hydrophilic regions of the protein. It is also useful to consider the predicted secondary structure of the protein, and direct antibodies to loop regions, i.e., non-α helical, non-β sheet regions. These regions have an inherent greater atomic mobility, and such flexibility is thought to be advantageous for the crossreaction of antipeptide antibodies with protein antigen. Such regions can be

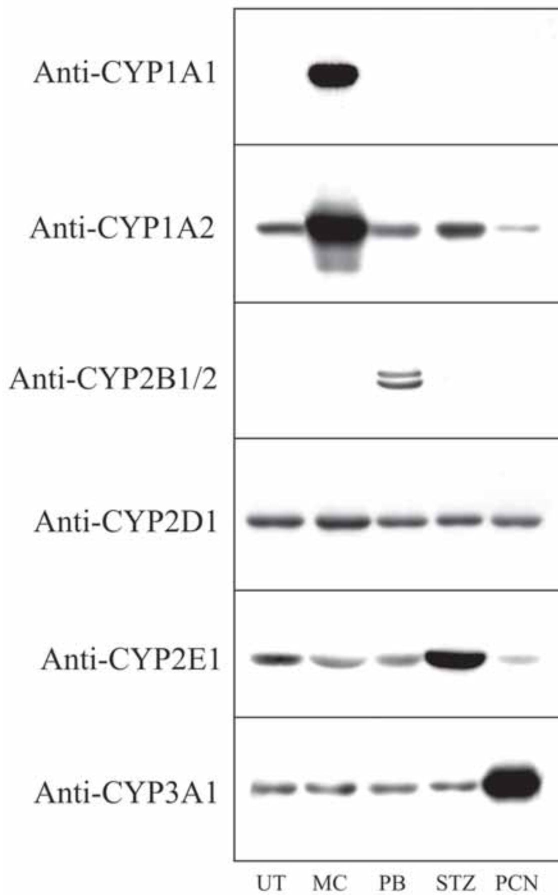


Fig. 1. Series of immunoblots demonstrating selectivity of binding of various antipeptide antibodies targeted against rat P450 enzymes. Antibodies were raised against the C-terminus of rat CYP1A1 (QHLQA), CYP2D1 (REQGL), CYP2E1 (VIPRS), and CYP3A1 (IITGS), and against internal sequences of CYP1A2 (TGALFKHSENYK; residues 283–294) and to a region common to CYP2B1 and CYP2B2 (IDTYLLRMEKEK; residues 265–276 of CYP2B1 and CYP2B2). Liver microsomal fractions were prepared from rats that were either untreated (UT) or treated with 3-methylcholanthrene (MC), sodium phenobarbital (PB), streptozotocin (STZ), or pregnenolone 16α-carbonitrile (PCN) as described previously *(13,14)*. To each lane 10 μg of microsomal protein was applied except for immunoblots developed with anti-CYP2B1/2 and anti-CYP3A1 antibodies where 5 μg of microsomal protein was used. After electrotransfer onto nitrocellulose filters, each blot was developed with antiserum diluted 1:4000 except for the anti-CYP3A1 antiserum, which was used at a dilution of 1:16,000. Only the central section of blots corresponding to the mol wt range of 50–60 kDa is shown. No other immunoreactive bands were detected.

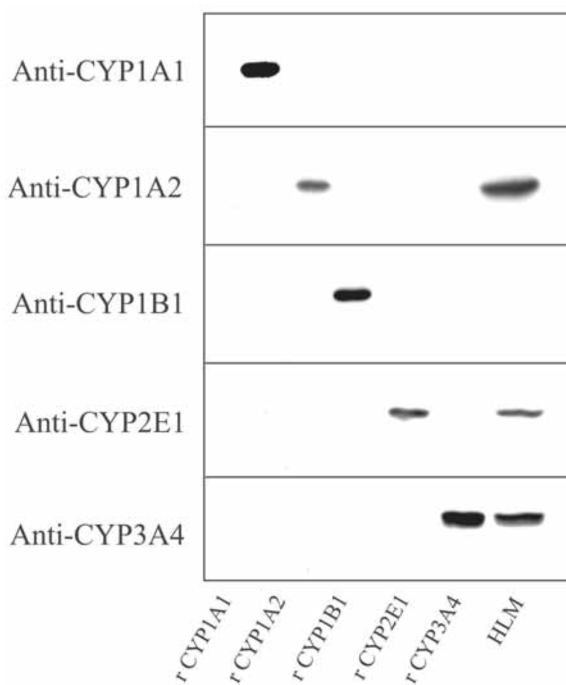


Fig. 2. Series of immunoblots demonstrating selectivity of binding of various antipeptide antibodies targeted against human cytochrome P450 enzymes. Antibodies were raised against the C-terminus of human CYP1A1 (MQLRS), CYP1B1 (KETCQ), CYP2E1 (VIPRS), and CYP3A4 (TVSGA), and to an internal sequence of CYP1A2 (TGALFKHSKKGPR; residues 284–296). The binding of antibodies to insect cell microsomal fractions containing recombinant human CYP1A1 (rCYP1A1; 2 µg) or CYP1B1 (rCYP1B1; 10 µg), and lymphoblastoid cell microsomal fractions containing recombinant human CYP1A2 (rCYP1A2; 10 µg), CYP2E1 (rCYP2E1; 5 µg) or CYP3A4 (rCYP3A4; 10 µg), was compared with binding to a pool of six samples of human liver microsomal fraction (HLM, 25 µg). Microsomal fractions containing recombinant enzymes were obtained from Gentest (Woburn, MA). After electrotransfer onto nitrocellulose filters, each blot was developed with antiserum diluted 1:4000. Only the central section of blots corresponding to the mol wt range of 50–60 kDa is shown. No other immunoreactive bands were detected.

identified using Web-based programs available on a variety of sites, including the ExPASy Proteomics Server at http://ca.expasy.org/ and the Pôle BioInformatique Lyonnais Gerland site at http://pbil.ibcp.fr/html/pbil_index.html. Good success with this approach has been found, but it should be recognized that although antibodies that bind to peptide are almost always produced, their crossreaction with protein is not certain. However, we have found that the affinity and potency of such antibodies can be improved if the immunizing peptide is cyclized *(12)*.

3. One of the advantages of using the antipeptide approach is that antibodies can be directed to predetermined sites on the surface of P450. This allows studies of the function of P450 enzymes by directing to regions on the surface of the P450 enzymes antibodies that interfere with catalytic activity ***(11,12)***. To raise such antibodies, it is useful to consider models of the three-dimensional structure of eukaryotic P450 enzymes ***(15)***.
4. Whatever use is intended for the antibody, an important issue is likely to be the specificity of binding. This property can be predetermined by directing antibodies to sequences that are unique to one form of P450. Although in many cases the choice may be clear, it is possible that similar, but not identical, sequences occur in other P450 enzymes. It is difficult to devise guidelines to deal with this gray area. However, in the case of C-terminally directed antibodies, the C-terminal residue is often critical for antibody binding, and alteration of this amino acid alone can greatly reduce antibody binding ***(2)***. The sequence of the selected peptide should also be compared with the sequences of proteins other than P450. A complete search of sequences in protein sequence databases can be performed using computer programs such as BLAST or FASTA, which are accessible through the National Center for Biotechnology Information's Web site at www.ncbi.nlm.nih.gov/BLAST/ or the Pôle BioInformatique Lyonnais Gerland site at http://pbil.ibcp.fr/html/pbil_index.html.
5. The size of the peptide used to raise antibodies should also be considered. When raising antibodies against the C-terminus of P450 enzymes, I have mostly used peptides representing the last five amino acid residues of the protein, with the addition of cysteine to the N-terminus for the purpose of conjugation to carrier protein. However, for several P450 enzymes in the CYP2B, CYP2C, and CYP2D subfamilies, cysteine is already present as the fifth residue from the C-terminus. In these cases, the naturally occurring cysteine was used for coupling to the carrier protein. This effectively reduced the immunizing sequence to a four amino acid residue peptide. Nevertheless, antibodies raised against these peptides also bound successfully to their target P450 enzymes. In human CYP1B1, the C-terminus contains cysteine in the penultimate position. This problem was overcome by synthesizing a hexapeptide containing two cysteine residues with different side chain–protecting groups. At the N-terminus, a trityl cysteine was used (as in most other syntheses), but the cysteine in the penultimate position was protected with a *t*-butylthio group. Deprotection and cleavage of the peptide from the solid-phase resin was performed for 2 h in 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane. Under these conditions, the thiol of the cysteine residue at the N-terminus was unmasked, whereas the *t*-butylthio-protected cysteine residue remained protected. After coupling of the peptide to KLH, the *t*-butylthio group was removed by treatment with 0.2 *M* 2-mercaptoethanol. The conjugate was then dialyzed against 0.05 *M* NaCl, 0.1 *M* NaCl, 1 m*M* EDTA, 1 m*M* dithiothreitol, pH 7.4. Immunization with this conjugate resulted in antibodies that bound to human CYP1B1 (*see* **Note 18**).
6. Regarding targeting internal sequences of P450 enzymes, peptides between 7 and 12 amino acid residues have been used successfully. Although it is tempting to synthesize peptides as large as possible, this is often restricted by considerations of similarity with other P450 enzymes. Thus, a longer peptide may have a better chance of producing antibodies that bind to the target P450, but if the peptide contains regions that occur in other P450 enzymes, then the resultant antibodies may also bind to these forms.
7. Details of the methods for the synthesis of peptides have been described in a previous volume of this series ***(16)*** and elsewhere ***(17,18)***. I have employed *N*-α-9-fluorenylmethoxycarbonyl (Fmoc) chemistry using manual, semiautomated and fully automated pep-

tide synthesizers to make peptides on solid-phase resins. I recommend the use of 4-benzyloxybenzyl alcohol (Wang resin) or 2-chlorotrityl resins. For convenience, these resins were purchased with the first (C-terminal) amino acid already coupled. Fmoc-protected amino acids were activated using benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate; alternatively, preformed activated pentafluorophenyl esters were used, except for serine and threonine, which were coupled as 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine esters. Typically, cysteine was added to the N-terminus of peptides for the purpose of conjugation to proteins. Peptides were cleaved from the resin, and side chain-protecting groups removed from amino acids, by treatment for 2 h with 95% TFA and 5% ethanedithiol. This mixture is effective in scavenging trityl groups liberated from cysteine and preserving the thiol group. After removal of TFA by rotary evaporation, the peptide was precipitated by the addition of ice-cold diethyl ether. The peptide was sedimented by centrifuging (250*g* for 10 min), the supernatant was removed, and the precipitated peptide was washed a further three times in a similar manner. Finally, the peptide was dried under a stream of nitrogen and dissolved in 5–10 mL of 0.5 *M* acetic acid before being lyophilized. Occasionally, a peptide was insoluble in this solution. In such cases, the suspension was lyophilized and small portions of the peptide were tested for solubility in other solvents, such as glacial acetic acid or DMF. Often, once dissolved, the solution may be successfully diluted into 0.5 *M* acetic acid. If it is necessary to use neutral or alkaline conditions to dissolve the peptide, precautions should be taken to avoid oxidation of the thiol group, such as the addition of EDTA and degassing of solutions.

8. Peptides were purified by gel filtration using a Sephadex G-15 column (30 × 1.5 cm) in 0.5 *M* acetic acid with a flow rate of 1 mL/min. Fractions of 4 mL were collected and analyzed for the presence of thiopeptide through the use of DTNB. The purity of each thiol-containing fraction was assessed by high-performance liquid chromatography (HPLC). HPLC was performed on a Nucleosil C18 10-µm column (Jones Chromatography, Hengoed, UK) with constant monitoring of the eluent at 210 nm. The gradient employed was typically 0–25% (v/v) acetonitrile (containing 0.1% [v/v] TFA) over 10 min at a flow rate of 2 mL/min, but this gradient can be extended for more hydrophobic peptides. Typically, products of >90% purity were obtained. Most commonly, impurities are peptides with side chain-protecting groups attached (because they were either not removed or reattached during the deprotection reaction). Owing to the strong ultraviolet absorbance of such groups, the amount of impurity is often overestimated. Frequently, impurities can be removed or reduced to an acceptable level by a gel filtration chromatography step, probably as a result of increased hydrophobic interaction of the impurity. Thus, impurities are usually retarded during the chromatography. Fractions containing thiopeptide with the greatest purity were pooled and lyophilized. Further purification of these small hydrophilic peptides was rarely necessary. The composition of each peptide was confirmed by electrospray mass spectrometry.
9. If solubility problems are encountered during the synthesis, it is possible that the peptide will not dissolve in 0.05 *M* sodium phosphate buffer, pH 6.0. Attempts should be made to dissolve the peptide as described in **Note 7**. If this proves ineffective, it is still possible to measure the thiol content of the suspension, provided that the peptide is soluble in the alkaline buffer used for this purpose. Often, such peptides can still be successfully coupled.
10. To ensure a good immunological reaction, it is usually necessary to conjugate peptides to a carrier protein. KLH is the carrier protein of choice for immunizations. This protein is strongly immunogenic, and antibodies against KLH do not crossreact with mammalian

proteins. The preparations available from Calbiochem are soluble in 0.1 *M* phosphate buffer; however, KLH obtained from other suppliers should be tested for solubility, because some have been found to contain very little soluble material. Furthermore, note that in low-ionic-strength buffers, KLH will precipitate out of the solution.

11. The use of MBS for coupling thiopeptide to carrier protein results in a complex with peptide coupled in a specific orientation. This also avoids masking of potentially antigenic groups in the peptide. Other crosslinking reagents, such as glutaraldehyde and carbodiimide, have been used successfully, but these reagents often crosslink through the reactive side chains of amino acids, i.e., the ε-amino group of lysine or the carboxylic acid group of aspartate and glutamate residues.
12. In some cases, the resulting peptide-KLH conjugate may become cloudy as a result of some precipitation. Such conjugates appear to be equally effective immunogens as soluble conjugates.
13. The degree of conjugation of peptide to KLH can be assessed by comparing the amino acid analysis of conjugated and unconjugated KLH. Typically, a ratio of 0.2 μmol of peptide/mg of carrier protein is achieved. Although not quantitative, it is more convenient to assess the success of the conjugation reaction by simultaneously coupling peptide to lysozyme under the same conditions used to couple peptide to KLH. Because lysozyme has a relatively low molecular weight, the result of the coupling reaction can be easily assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% (w/v) polyacrylamide gels. Successful conjugation is evident as an increase in the size of lysozyme, usually as a series of bands of increasing molecular weight.
14. When antiserum is raised against a peptide representing the C-terminus of a P450 enzyme, antibodies of the required specificity and binding strength are usually produced in the first bleed, and subsequent booster injections have little effect on the binding characteristics. However, antibodies raised against peptides that represent internal sequences of P450 enzymes tend to be more variable in their binding to P450 enzymes. Often antibodies with the desired binding characteristics can be produced in the first blood sample, but it is not uncommon for the best antibodies to be produced after one or more booster injections. Occasionally, the converse may occur and antibodies of the desired characteristics are lost with booster injections. It is important, therefore, to assess antisera as soon as possible after collection and decide whether to continue or terminate the immunization procedure.
15. Most of this work has been performed using rabbits and sheep; however, other species may also be used for the production of antipeptide antibodies.
16. Immunoblot analysis is particularly useful in determining the specificity of binding of the antibodies produced. I have used SDS-PAGE with 9% gels to separate microsomal proteins and electroblotting to transfer the proteins onto nitrocellulose filters ***(19,20)***. Nonspecific binding sites on the filter are blocked by incubation in 3% (w/v) bovine serum albumin (BSA) in PBS for 1 h at room temperature or 4°C overnight. After rinsing in 50 mL of PBS, filters are incubated for 1 h at room temperature in an appropriate dilution of antiserum (e.g., 1:1000–1:16,000) in 20 mL of 0.1% (w/v) BSA in PBS. The filter is washed five times with about 25 mL of washing solution (PBS containing 0.05% [w/v] BSA and 0.05% [v/v] Tween-20) each time and then incubated for 1 h at room temperature in 20 mL of goat antirabbit IgG-peroxidase conjugate diluted 1:25,000 in 0.1% (w/v) BSA in PBS. Afterword, the filter is washed five times as previously described. Immunoreactivity is detected using enhanced chemiluminescence following the instructions supplied by the manufacturer (Amersham plc, Little Chalfont, UK).

17. For antirat P450 antibodies, specificity can be demonstrated by comparing the binding to liver microsomal membrane proteins isolated from rats treated with various cytochrome P450-inducing compounds (**Fig. 1**). Thus, the anti-CYP1A1 antibody bound to a single band in microsomal membrane proteins isolated from rats treated with 3-methylcholanthrene, but not to microsomal membrane proteins isolated from any of the other rats. The anti-CYP1A2 antibody bound to a single band in microsomal proteins isolated from all the rats, and the intensity of the immunoreactive band was greatly increased in those treated with 3-methylcholanthrene. The anti-CYP2B1/2 antibody bound only to microsomal proteins from rats treated with sodium phenobarbital, where two bands, corresponding to CYP2B1 and CYP2B2, were detected. The anti-CYP2D1 antibody bound to a single band with similar intensity in all rats, regardless of their treatment. The anti-CYP2E1 antibody bound to a single band in microsomal proteins from all rats, and the intensity of the immunoreactive band was increased in those treated with streptozotocin. Finally, the anti-CYP3A1 antibody bound to a single band in microsomal proteins from all rats, and the intensity of the immunoreactive band was very strongly increased after treatment with pregnenolone 16α-carbonitrile. These results are consistent with the known pattern of expression of these P450 enzymes on exposure of rats to the inducing agents.
18. For the antihuman P450 antibodies, specificity can be assessed by examining the binding to recombinant human P450 enzymes. In the examples shown (**Fig. 2**), each of the antihuman P450 antibodies bound only to their respective recombinant P450 antigens. Antibodies targeted against CYP1A2, CYP2E1, and CYP3A4, but not those targeted against CYP1A1 or CYP1B1, bound to human liver microsomal membrane proteins, consistent with the expression of these P450 enzymes in normal human liver.

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# 23

## The Human Cytochrome P450 Allele Nomenclature Committee Web Site

### *Submission Criteria, Procedures, and Objectives*

**Sarah C. Sim and Magnus Ingelman-Sundberg**

**Summary**

Interindividual variability in xenobiotic metabolism and drug response is extensive. Genetic factors are predicted to account for 15–30% of this variability in general, but for certain drugs the genetic factor is the major determinant for outcome of drug therapy. Of particular importance for drug metabolism, drug response, and adverse drug reactions are the cytochrome P450 (CYP) enzymes, many of which are polymorphic. An essential basis for research and applications regarding interindividual variability in xenobiotic metabolism and toxicity by polymorphic CYPs is to have a common nomenclature for genetic variants and a system that allows researchers to be rapidly updated within the field. Since 1999 this has been achieved by the operation of the Human Cytochrome P450 Allele Nomenclature Committee Web site (www.imm.ki.se/CYPalleles/), where novel allelic variants are published after peer review. Currently, this Web site covers the nomenclature for polymorphic alleles of 22 CYP isoforms including more than 200 functionally different variants. Each CYP has its own Web page, which lists the alleles with their nucleotide changes, their functional consequences, and links to publications where the allele has been identified and characterized. The CYP allele Web site offers a rapid on-line publication of new alleles, provides an overview of peer-reviewed data, and serves as a form of quality control on research on new alleles.

**Key Words**: Pharmacogenetics; adverse drug reactions; drug response; haplotypes; drug metabolism.

## 1. Introduction

Interindividual variability in xenobiotic metabolism and drug response is extensive. Drug concentrations in plasma can vary more than 600-fold between two individuals with the same weight and using the same drug dosage. The causes for this interindividual variation can be of genetic, physiological, pathophysiological, or environmental origin.

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Genetic explanations for interindividual variability have been demonstrated for drug absorption, drug metabolism, and drug-target interactions. This forms the basis for slow or rapid drug absorption; poor, efficient, or ultrarapid drug metabolism; and poor or efficient drug-target interactions. One can envision that genetic factors account for about 15–30% of the interindividual differences in drug metabolism and response in general, but for certain drugs or classes of drugs, the genetic factor is the sole, or at least the main, determinant for outcome of drug therapy ***(1–3)***. Furthermore, the interindividual difference in susceptibility to carcinogens and environmental toxins is extensive, and polymorphic enzymes play a key role in their bioactivation and inactivation. Of particular importance for drug metabolism, drug response, and susceptibility to toxic compounds are the cytochrome P450 (CYP) enzymes,which are responsible for 75–80% of all phase I-dependent drug metabolism.

A meta-analysis by Lazarou et al. ***(4)*** revealed that serious adverse drug reactions (ADRs) occur in 6.7% of all hospitalized patients and that 0.3% of all hospitalized patients develop fatal adverse reactions, thus resulting in more than 100,000 deaths annually in the United States. Of 1232 chemical entities approved as drugs in the United States, 16% are associated with ADRs requiring a warning on the product label ***(5)***. It has been estimated that ADRs cause up to 7% of all hospital admissions in the United Kingdom ***(6)*** and that 13% of all admissions to internal medicine clinics in Sweden are owing to ADRs ***(7)***. Adverse reactions to anticoagulant, anti-inflammatory, and cardiovascular drugs represent severe but preventable predicaments in drug treatment that collectively make up more than half of all ADRs. The cost of ADRs, including on average 2 d of prolonged hospitalization and reduced productivity, has been estimated to amount to $75–100 billion annually in the United States ***(8)***. In addition, the efficiency of drug treatment is far from optimal; the general response rate in drug treatment is between 20 and 60%.

The genetics of drug-metabolizing enzymes, in particular CYP is a critical aspect in the design of improved drug therapies with higher response rates and reduced ADRs. In addition, the genetics of CYP enzymes is taken into great consideration at an early stage when new drug candidates are developed in the pharmaceutical industry. A fundamental basis for the research and applications regarding interindividual variability in xenobiotic metabolism by polymorphic CYPs is to have a common nomenclature for genetic variants and a system that allows researchers to be rapidly updated within the field.

## 2. Cytochromes P450

There appear to be 57 active *CYP* genes (http://drnelson.utmem.edu/cytochromeP450.html) and 58 pseudogenes in the human genome ***(9)***. The majority of *CYP* genes that encode enzymes involved in the metabolism of drugs and other xenobiotics are polymorphic. Mutations in *CYP* genes can lead to abolished, reduced, altered, or increased enzyme activity. Abolished enzyme activity is commonly seen in cases in which the whole gene has been deleted but also has its origin in mutations causing altered splicing, introduction of stop codons, abolished transcriptional start sites, and deleterious amino acid changes. Mutations in substrate recognition sites can result in the synthesis

of enzymes with altered substrate specificity. Increased enzyme activity can be the result of mutations that affect the transcription rate of *CYP* genes. In addition, duplications of active *CYP* genes occur, leading to increased drug metabolism and the ultrarapid metabolizer phenotype.

CYP is the main class of enzymes responsible for the metabolism of drugs and other xenobiotics. CYP enzymes are responsible for 75–80% of the phase I–dependent metabolism and 65–70% of the clearance of clinically used drugs ***(10,11)***. Whereas no quantitatively important polymorphism has been described for CYP3A4, an enzyme that accounts for about 50% of all CYP-dependent drug metabolism, CYP2C9, CYP2C19, and CYP2D6 are highly polymorphic and collectively account for about 40% of the metabolism of clinically used drugs. In addition, CYP1A2, CYP2A6, and CYP2B6 are polymorphic enzymes that significantly contribute to xenobiotic metabolism. Knowledge about polymorphisms in these genes can provide important information for drug dosage and choice of drug therapy ***(1–3,12,13)***. The polymorphic background of CYPs is frequently responsible for the development of adverse drug reactions. According to Phillips et al. ***(14)***, 56% of drugs that are cited in ADR studies are metabolized by polymorphic phase I enzymes, of which 86% are CYPs. By comparison, only 20% of drugs that are substrates for nonpolymorphic enzymes are mentioned in the studies on ADR. In addition, the cost of treating patients who have polymorphic variants of CYPs is considerably higher than for those who do not have polymorphic alleles ***(8)***. Based on a meta-analysis by Kirchheiner et al. ***(3)*** regarding treatment with antipsychotics and antidepressants, it is evident that dosage based on *CYP2D6* and *CYP2C19* genotype is relevant for about 50% of clinically used drugs that are substrates for these enzymes.

## 3. The Human Cytochrome P450 Allele Nomenclature Committee Web Site

### *3.1. Introduction*

In recent years, a substantial amount of research in the CYP area has focused on the identification and characterization of polymorphic human *CYP* genes. The rapid development in the field required the establishment of a Web site with recommended nomenclature regarding the various allelic variants of CYPs. The aims were to encourage scientists worldwide to speak the same language and to avoid “homemade” allelic designations that can confuse the nomenclature system and the scientific literature. In addition, rapid on-line publication and summary of alleles would prevent unnecessary duplication of research efforts aimed at characterizing alleles already described. For this purpose, an initiative was taken in 1999 to create the Human Cytochrome P450 Allele Nomenclature Web site (www.imm.ki.se/CYPalleles/) at a server at the Karolinska Institutet, with Mikael Oscarson as the first Webmaster, and Sarah C. Sim as his successor in February 2004. An editorial board of the Allele Nomenclature Committee consisting of Magnus Ingelman-Sundberg, Ann K. Daly, and Daniel W. Nebert was established as well as an international advisory board, with Jürgen Brockmöller, Michel Eichelbaum, Seymour Garte, Joyce A. Goldstein, Frank J. Gonzalez, Fred F. Kadlubar, Tetsuya Kamataki, Urs A. Meyer, David R. Nelson, Michael R. Waterman, Anna Wedell, and Ulrich M. Zanger as members. The nomenclature system chosen for

the *CYP* allele Web site was based on guidelines for human gene nomenclature by Shows et al. *(15)*; the system for allelic designations for *CYP2D6* described by Daly et al. *(16)*; recommendations for a nomenclature system for human gene mutations, as proposed by Antonarakis and the Nomenclature Working Group *(17)*; and recommendations for the description of human sequence variations by den Dunnen and Antonarakis *(18)*.

Currently, the Web site covers the nomenclature for polymorphic alleles of 22 CYP enzymes: CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A13, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP2R1, CYP2S1, CYP3A4, CYP3A5, CYP3A7, CYP3A43, CYP4B1, CYP5A1, CYP8A1, and CYP21A2. Each isoform has its own Web page, which lists the alleles with their nucleotide changes, their functional consequences and links to publications identifying or characterizing the alleles (**Fig. 1**). The *CYP* allele Web site is widely used in research and drug development by academic researchers and the drug industry. It has had more than 100,000 visits since February 21, 2001, and there are currently more than 600 visits per week. The visitors come from Asia, Europe, and the United States at an almost equal representation and about 10% are from companies. The aim, nomenclature system, and features of the Web site have been described in letters to *Pharmacogenetics* *(19)* and *Cancer Epidemiology, Biomarkers and Prevention* *(20)*.

### *3.2. Inclusion Criteria for* CYP *Alleles*

The *CYP* allele Web site takes all allelic variants into account and describes sequence variations in the coding region, introns and regulatory regions of the *CYP* genes. The designation of an allele ideally requires determination of all sequence variations in a gene, i.e., the complete haplotype. On the Web site, a gene is considered as the sequence from 5 kb upstream from the transcription start site to 500 bp downstream of the last exon. However, if a regulatory element has been characterized at a more distant part of the gene, it too is considered to belong to the gene.

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Fig. 1. *(opposite page)* A representative webpage of the Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee website. A link to the reference sequence regarded as the **1A*-allele is either provided beneath the table (Genbank) or inserted under the headline for the column Nucleotide changes. The intention is to provide both cDNA and genomic reference sequences for all CYPs present on the website. When both cDNA and genomic reference sequences are available on a webpage, the numbering of sequence variations in the alleles is provided for both of them. The allelic names are listed in the first column, followed by the protein designation, which follows the numeric name of the allele. Under Nucleotide changes, all known sequence variations in the allele are listed. The sequence variations leading to the most severe functional consequences are given in **Bold** (not yet for all CYPs). By clicking underlined SNPs a direct connection to the corresponding dbSNP http://www.ncbi.nlm.nih.gov/SNP/ is obtained. In the Effect column, the consequences of the sequence variations are listed, such as amino acid changes, frameshifts, splicing defects, inserts of stop codons, and so on. Enzyme activity characterizations are presented for both in vivo and in vitro studies when available. The last column provides links to references where the allele has been identified or characterized.

## CYP2C8 allele nomenclature

| *Allele* | *Protein* | *Nucleotide changes, cDNA* | *Trivial name* | *Effect* | *Enzyme activity* | | *References* |
|---|---|---|---|---|---|---|---|
| | | | | | *In vivo* | *In vitro* | |
| *CYP2C19*1A* | CYP2C19.1A | None | | | Normal | Normal | Romkes *et al*, 1991 |
| *CYP2C19*1B* | CYP2C19.1B | 99C>T; 991A>G | | I331V | Normal | | Richardson *et al*, 1997 |
| *CYP2C19*1C* | CYP2C19.1B | 991A>G | | I331V | Normal | | Blaisdell *et al*, 2002 |
| *CYP2C19*2A* | | 99C>T; **681G>A**; 990C>T; 991A>G | m1; m1A | **splicing defect**; I331V | None | | de Morais *et al*, 1994a |
| *CYP2C19*2B* | | 99C>T; 276G>C; **681G>A**; 990C>T; 991A>G | m1B | E92D; **splicing defect**; I331V | None | | Ibeanu *et al*, 1998b |
| *CYP2C19*3* | | **636G>A**; 991A>G; 1251A>C | m2 | **W212X**; I331V | None | | de Morais *et al*, 1994b |
| *CYP2C19*4* | | **1A>G**; 99C>T, 991A>G | m3 | **GTG initiation codon**; I331V | None | | Ferguson *et al*, 1998 |
| *CYP2C19*5A* | CYP2C19.5A | **1297C>T** | m4 | **R433W** | None | None | Xiao *et al*, 1997 Ibeanu *et al*, 1998a |
| *CYP2C19*5B* | CYP2C19.5B | 99C>T; 991A>G; **1297C>T** | | I331V; **R433W** | None | | Ibeanu *et al*, 1998a |
| *CYP2C19*6* | CYP2C19.6 | 99C>T; **395G>A**; 991A>G | m5 | **R132Q**; I331V | None | None | Ibeanu *et al*, 1998b |
| *CYP2C19*7* | | **IVS5+2T>A** | | **splicing defect** | None | | Ibeanu *et al*, 1999 |
| *CYP2C19*8* | CYP2C19.8 | **358T>C** | | **W120R** | None | Decr | Ibeanu *et al*, 1999 |
| *CYP2C19*9* | CYP2C19.9 | 99C>T; **431G>A**; 991A>G | | **R144H**; I331V | | (Decr) | Blaisdell *et al*, 2002 |
| *CYP2C19*10* | CYP2C19.10 | 99C>T; **680C>T**; 991A>G | | **P227L**; I331V | | Decr | Blaisdell *et al*, 2002 |
| *CYP2C19*11* | CYP2C19.11 | 99C>T; 449G>A; 991A>G | | R150H; I331V | | | Blaisdell *et al*, 2002 |
| *CYP2C19*12* | CYP2C19.12 | 99C>T; 991A>G; **1473A>C** | | I331V; **X491C**; 26 extra aa | | Unstable | Blaisdell *et al*, 2002 |
| *CYP2C19*13* | CYP2C19.13 | 991A>G; 1228C>T | | I331V; R410C | | | Blaisdell *et al*, 2002 |
| *CYP2C19*14* | CYP2C19.14 | 50T>C; 99C>T; 991A>G | | L17P; I331V | | | Blaisdell *et al*, 2002 |
| *CYP2C19*15* | CYP2C19.15 | 55A>C; 991A>G | | I19L; I331V | | | Blaisdell *et al*, 2002 |
| *CYP2C19*16* | CYP2C19.16 | 1324C>T | | R442C | | | Morita *et al*, 2004 |
| | | 221T>C | | M74T | | | Solus *et al.*, 2004 |
| | | 502T>C | | F168L | | | Solus *et al.*, 2004 |
| | | 636G>T/C/A | | W212C/C/X | | | NCBI dbSNP |

**Additional SNPs, where the haplotype has not yet been determined**

The *CYP* allele Web site attempts to serve as a form of quality control by demanding a well-undertaken characterization of new alleles. For assignment of a new allele, an effect on enzyme activity does not need to be proven, but the allele is required to be well characterized regarding linkage or lack of linkage to an identified polymorphism with other nucleotide variations. This includes sequencing of all exons and intron-exon junctions and, optimally, also the 5'- and 3'-flanking regions. In general, genotyping for only certain polymorphic sites without characterizing the rest of the gene and construction of haplotypes using computer programs are not accepted. Such characterizations have been included on the Web site but are generally indicated as being incomplete.

To receive a unique allelic number (e.g., *CYP2B6*4*), the nucleotide changes should affect transcription, splicing, translation, or posttranscriptional or posttranslational modifications or result in at least one amino acid change. If the effective nucleotide change also exists together with other nucleotide changes not shown to have any effect, this allele will be defined by an additional letter (e.g., *CYP2B6*4B*), whereas the primary allele will be designated by an *A* (e.g., *CYP2B6*4A*). When several effective polymorphisms are present on the same allele, the allelic number given is based on the polymorphism that causes the most severe functional consequence. For example, if a polymorphism that gives rise to a splicing defect (e.g. *CYP2C19*2A*) also exists in combination with other less effective mutations such as amino acid changes, this allele will receive an additional letter (e.g., *CYP2C19*2B*). If an allele is composed of a combination of polymorphisms none of which has been shown to lead to a severe consequence on its own but still has an impact on the protein, such as amino acid substitution, the combination allele will be given a new numeric allele name (e.g., *CYP2B6*6*). The first allele sequenced is designated the reference allele and carries the allele name **1* (or **1A*). Thus, the **1* allele may not be the major allele in every ethnic group. There are some inconsistencies regarding the allelic names of early characterized genes that do not follow today's guidelines (**Table 1**). Renaming of alleles would result in confusion and has been avoided. Thus, alleles incorporated at an early stage do not always follow the nomenclature system.

### 3.3. Procedures for Submission of New Alleles

New alleles should be submitted to the Webmaster (Sarah.Sim@imm.ki.se) together with information sufficient to satisfy the criteria to be assigned an allele (**Table 1**; www.imm.ki.se/CYPalleles/criteria.htm). For incorporation into the Web site as an allele, all exons and exon-intron junctions need to be sequenced, and haplotype analysis needs to be done experimentally if other polymorphisms are shown to coexist. If a new allele has been detected at the cDNA level, verification at the genomic level is required. New variants are frequently submitted to the *CYP*-allele Web site. Most submissions concern reservation of allelic names for novel variants intended for publication in a journal or on-line. It is highly recommended that the Webmaster be contacted to reserve an allelic name to be included in manuscripts before submission and publication. The use of allele names that have not been approved by the Allele Nomenclature Committee is strongly discouraged, as is the use of invented nomenclature systems using the *-function

**Table 1**
**Criteria for Inclusion of Alleles in the Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee Web Site**

1. Only human CYP alleles are considered.
2. The gene and allele are separated by an asterisk followed by Arabic numerals and upper-case Roman letters with less than four characters to name the allele (e.g., *CYP1A1*3*, *CYP1B1*22*, *CYP2D6*10B*).
3. A gene is considered as the sequence from 5 kb upstream of the transcription start site to 500 bp downstream of the last exon. However, if a regulatory element has been characterized at a more distant part of the gene, this area also belongs to the gene.
4. For an allele to be assigned as unique, it should contain nucleotide changes that have been shown to affect transcription, splicing, translation, or posttranscriptional or posttranslational modifications or result in at least one amino acid change.
5. Additional nucleotide changes and combinations of nucleotide changes in the gene will be given letters (e.g., **21A*, **21B*). Thus, in cases in which silent mutations occur or mutations are present in regulatory parts or introns with unclear function, the allelic name should adhere to the closest functionally characterized allele by subgroup assignments such as *CYP2D6*4A*. Allelic variants can be defined as combinations of up to three letters (e.g., *CYP2D6*2ABC*), thereby allowing room for 22 × 22 × 22 = 10,648 different variants for each allelic number. The letters *I, O, X,* and *Y* are excluded because of indexing problems.
6. For extra gene copies (*n*) placed in tandem, the entire allelic arrangement should be referred to as, e.g., *CYP2D6*2Xn*.
7. Numbering of nucleotides in the allele should be as described in **ref. *16***. The base A in the initiation codon ATG is denoted +1 and the base before A is numbered –1.
8. For reasons of indexing, the names for proteins should have a period between the name of the gene product and number (e.g., CYP2D6.2A).
9. The wild-type allele is defined as the sequence of the first alleles sequenced and should be designated as **1* (or **1A* and **1B* in the case of slightly variant sequences).
10. Single-nucleotide polymorphisms (SNPs) that are not easily assigned to a specific allele will be listed at the bottom of the corresponding nomenclature site with relevant literature references.
11. New alleles should be submitted with information sufficient to satisfy the criteria for being assigned a unique allele, as under no. 4, or a letter, as described under no. 5. For incorporation into the Web site as a unique allele, all exons and exon-intron borders should be sequenced. If a new allele has been detected at the cDNA level, verification of the mutation(s) at the genomic level is required. For acceptance of a new SNP given a separate letter, evidence for its presence at the genomic level is required.
12. No temporary allelic numbers or letters are provided, and information about any new allele submitted will continuously be published on the Web site. In case an author does not wish to release the information on the Web site before publication, the Webmaster can usually provide him or her with an allelic designation but not release the information on the Web site until the manuscript has been accepted or published.

(e.g., **IIX*). If an allelic name has not been reserved, authors should simply refer to novel alleles in their manuscript as allele A or allele 1, and so forth. If manuscripts/papers are provided in which new information about an already existing allele is presented, the literature reference will be added to the Web page in question. In cases in which the authors do not wish to release the information on the *CYP* allele Web site before publication, the Webmaster will provide them with an allelic designation but not release the information on the Web site or to any person until the manuscript has been accepted or published. This means that the Webmaster has many already reserved designations for new alleles that are not official, thus making it impossible for scientists to make their own designations based on the existing alleles published on the Web site.

After submission to the Webmaster of results showing the existence of new alleles, the data are evaluated. In certain cases members of the editorial and/or the advisory board are consulted. Often the Webmaster requires additional information, including original data, before the allele can be accepted for publication on the *CYP* allele Web site. Publication is accepted only when the inclusion criteria (*see* **Table 1**) are fulfilled. This makes the Web site unusual in its character, since only peer-reviewed data are published on it.

### *3.4. Problems Today and in the Future*

The number of submissions to the *CYP* allele Web site is increasing. A large number of sequence variations in introns and flanking regions as well as silent mutations in exons are submitted, partly as a consequence of the increasing number of genomic sequencing projects in different parts of the world. Yet the technology does not always allow an inexpensive and firm method for determining the exact haplotype of the variant gene submitted. Many haplotypes are suggested based on computer algorithms. At the Web site, one distinguishes between experimentally determined and computer-predicted haplotypes and specify the basis for their identification on the Web site. It is anticipated that in the future the true haplotypes will be more easily and accurately determined. Some allelic designations defined thus far will not represent the true content of sequence variations for that particular allele. In addition, a vast number of novel *CYP* haplotypes is expected in the future, which may necessitate a modified nomenclature system in due time.

## 4. Conclusion

The *CYP* allele Web site serves as an important platform for scientists in the CYP field by offering rapid on-line publication of new alleles and presenting a summary of known polymorphisms of *CYP* genes and their impact on enzyme function. In addition, the Web site serves as a form of quality control for the characterization of polymorphisms. Furthermore, new allelic variants of *CYP* genes are often first published on the Web site, making it of particular interest to researchers in this field.

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# 24

# Genotyping for Cytochrome P450 Polymorphisms

**Ann K. Daly, Barry P. King, and Julian B. S. Leathart**

**Summary**

Protocols for the extraction of DNA from human blood and for genotyping for a number of common cytochrome P450 polymorphisms using either polymerase chain reaction (PCR)-restriction fragment length polymorphism or PCR-single-strand conformational polymorphism (SSCP) analysis are described. Rapid high-throughput techniques are also available for analyses of this type, but they require access to specialized equipment and are not considered here. General guidelines for performing amplification using PCR are described together with electrophoresis protocols for analysis of restriction digests of PCR products with agarose and polyacrylamide gels including the use of polyacrylamide-based gels for SSCP analysis. Protocols for the following specific isoforms and alleles are also provided: CYP1A1 (**2B* and **4* alleles), CYP2C8 (**3* and **4* alleles), CYP2C9 (**2*, **3,* and **11* alleles), CYP2C19 (**2* and **3* alleles), CYP2D6 (**3*, **4*, **5,* and **6* alleles), CYP2E1 (**5A*, **5B,* and **6* alleles), and CYP3A5 (**3* allele).

**Key Words:** Polymerase chain reaction; restriction digest; polymorphism; CYP1A1; CYP2C8; CYP2C9; CYP2C19; CYP2D6; CYP2E1; CYP3A5.

## 1. Introduction

A large number of common functionally significant genetic polymorphisms in genes encoding certain cytochromes P450 (P450) have now been described and characterized (for reviews, *see* **refs.** ***1*** and ***2***). Individuals may lack certain enzyme activities or have higher or lower than normal activities owing to the presence of certain variant alleles. These individuals may be at altered risk of developing adverse drug reactions or diseases associated with xenobiotic exposure including cancer. Variant alleles can be detected by the direct approach of genotyping in which the individual's DNA is examined usually by an assay involving use of a polymerase chain reaction (PCR) or by phenotyping in which the individual's pattern of metabolites produced from a probe drug is examined.

In this chapter, we describe the detection of common polymorphisms in P450 genes relevant to drug and other xenobiotic metabolism using genotyping. Genotyping is now

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generally preferred to phenotyping because it is more convenient, requiring only a single blood sample that can be taken at any time. It can also be used on stored blood or other tissue samples. We also describe general methods for PCR and analysis of PCR products that have been used to genotype for pharmacogenetic polymorphisms together with the specific conditions used to detect a range of known polymorphisms in P450 genes.

The emphasis in this chapter is on genotyping methods using PCR-restriction fragment length polymorphism-based techniques, that can be undertaken without the need to purchase expensive specialist equipment. This issue is discussed in more detail elsewhere *(3)*, but if access to the most up-to-date medium- to high-throughput techniques such as pyrosequencing, real-time PCR or mass spectrometry systems (e.g., Sequenom) is available, these methods have the advantage of enabling thousands, rather than hundreds, of PCRs per week to be performed.

## 2. Materials

### *2.1. Preparation of DNA From Human Blood*

1. Human blood: collect 1–5 mL using either ethylenediaminetetra-acetic acid (EDTA) or citrate as anticoagulant. If required, the blood can be stored at –20°C for up to 1 yr or at –80°C for longer periods prior to DNA extraction.
2. Lysis buffer: 320 m*M* sucrose, 5 m*M* $MgCl_2$, 1% (v/v) Triton X-100, 10 m*M* Tris-HCl, pH 8.0. Make up the buffer without Triton X-100 and autoclave. Add Triton X-100 to the autoclaved solution while still warm, and immediately mix the solution vigorously to ensure uniform mixing. Store at 4°C.
3. Solution B: 150 m*M* NaCl, 60 m*M* EDTA, 1% (w/v) sodium dodecyl sulfate (SDS), 400 m*M* Tris-HCl, pH 8.0. Make up buffer without SDS and autoclave. Add SDS after cooling. Store at room temperature.
4. 5 *M* Sodium perchlorate: store at room temperature.
5. Chloroform precooled to –20°C.
6. Ethanol precooled to –20°C.
7. 70% (v/v) Ethanol.
8. 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.4: Autoclave. Store at room temperature.

### *2.2. Polymerase Chain Reaction*

1. Nucleotide mix (2 m*M* each of dATP, dGTP, dCTP, and dTTP): stock solutions of each of the four nucleotides are purchased from a commercial supplier (such as Roche, Lewes, UK) at a concentration of 100 m*M*, pH 7.0. To prepare 1.0 mL of 2 m*M* nucleotide mix, add 20 µL of each 100 m*M* nucleotide stock to 920 µL of sterile water and mix well. Dispense into 50-µL aliquots. Store at –20°C.
2. Thermostable DNA polymerase: these polymerases are available from a variety of suppliers, and we have obtained satisfactory results for the assays described here using enzyme from several different suppliers including Bioline (London, UK), Promega (Southampton, UK), and Molzyme GmbH (Bremen, Germany). We also use the Expand PCR system (Roche) in certain assays (*see* **Note 1**).
3. 10X PCR buffer: 500 m*M* KCl, 15 m*M* $MgCl_2$, 100 m*M* Tris-HCl, pH 9.0, 1% (v/v) Triton X-100. Autoclave the buffer prior to adding Triton X-100 and store in small aliquots at –20°C. Most suppliers of *Taq* polymerase will include a buffer with the enzyme although some types of PCR buffer can lead to problems with restriction digests (*see* **Note 2**).

4. Thermocycler.
5. PCR primers: the particular oligonucleotide primers required are provided for each assay. Primers are normally diluted to a concentration of 25 μ*M* in water and stored in small aliquots at –20°C. Satisfactory results are obtained from unpurified oligonucleotide preparations.

### 2.3. Gel Electrophoresis of PCR Products

1. 10X Tris-borate EDTA buffer (TBE): 0.9 *M* Tris-borate, 20 m*M* EDTA: to prepare 1 L, dissolve 108 g of Tris base and 55 g of boric acid in 900 mL of water. Add 40 mL of 0.5 *M* EDTA, pH 8.0. Make up the volume to 1 L with water. Autoclave. Store at room temperature.
2. Agarose: standard agarose gels may be prepared using molecular biology-grade agarose, which is available from a variety of suppliers.
3. 30% Acrylamide solution: dissolve 29 g of acrylamide and 1 g of *N,N′*-methylenebis-acrylamide to a final volume of 100 mL in water. **Caution:** Acrylamide is a neurotoxin and should be handled with great care especially when weighing. Acrylamide-bisacrylamide solutions are available from a variety of commercial suppliers and may be a more convenient and safe alternative to preparing the stock solution in the laboratory.
4. 10% (w/v) Ammonium persulfate.
5. Ethidium bromide (EtBr) solution: dissolve 0.1 g of solid EtBr in 10 mL of water. EtBr is a mutagen and must be handled with care. Store the solution at 4°C in a foil-wrapped universal container.
6. Molecular weight standards: when the products of a PCR reaction or restriction digest are of 1000 bp or less, a 100-bp DNA ladder is used (Invitrogen, Paisley, UK), which consists of DNA molecules in the range of 100–1500 bp and gives bands every 100 bp. When the products of the PCR reaction are of >1000 bp, we use λ DNA digested with the restriction enzyme *Hind*III, which gives bands of 23, 9.4, 6.6, 4.4, 2.3, and 2.0 bp, and 564 and 125 bp. This material is available commercially from a variety of suppliers.

### 2.4. Single-Strand Conformational Polymorphism Analysis

1. 10X Tris 2-(*N*-morpholino)ethanesulphonic acid (MES) EDTA buffer (TME): 0.3 *M* Tris, 0.35 *M* MES, 10 m*M* EDTA. To prepare 1 L, dissolve 36.3 g of Tris base, and 80.6 g of MES in 900 mL of water. Add 20 mL of 0.5 *M* EDTA, pH 8.0. Make up the volume to 1 L with water. The pH should be 6.8 without adjusting, but can be adjusted to 6.8 if necessary. Do not autoclave this solution.
2. 10% (w/v) Ammonium persulfate.
3. Proprietary Mutation Detection gel solution MDE (BioWhittaker, Rockland, ME) or SequaGel MD (National Diagnostics [UK], Hessle, Yorkshire, UK).
4. TEMED.
5. Urea.
6. Single-strand conformational polymorphism (SSCP) sample loading buffer: 95% (v/v) formamide, 0.01 *M* NaOH, 0.015% (w/v) xylene cyanol.
7. SYBR Gold (Molecular Probes, Leiden, The Netherlands): dilute 1 in 10,000 in 1X TBE buffer before use.

## 3. Methods

### 3.1. Preparation of DNA From Human Blood

1. Prepare nuclei by adding the blood sample to 35 mL lysis of buffer in a 50-mL conical polypropylene centrifuge tube. Mix gently and centrifuge at 2000*g* for 10 min at 4°C.

2. Resuspend the pellet in 2 mL of solution B and transfer to a 15-mL polypropylene centrifuge tube. To this add 0.5 mL of 5 *M* sodium perchlorate. Rotary mix the suspension at room temperature for 15 min and then incubate at 65°C for 30 min. Add 2 mL of chloroform and again rotary mix at room temperature for 10 min. Centrifuge at 1400*g* for 10 min to separate the phases.
3. Transfer the aqueous DNA-containing upper phase to a fresh tube and add 2 vol of ethanol. Invert the tube gently to precipitate the DNA and spool the DNA onto a disposable plastic loop. Wash the spooled DNA briefly in 70% ethanol and allow to dry at room temperature for 20 min.
4. Resuspend the DNA in 200–500 μL (depending on the initial volume of the blood used) of 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.4, by incubating at 60°C for 8–16 h, and store at 4°C until required.
5. Assess the yield, concentration, and purity of the DNA spectrophotometrically. The absorbance at 260 and 280 nm is determined typically for a 1-in-50 dilution of the DNA. The $A_{260}$ nm is used to calculate the DNA concentration and both $A_{260}$ and $A_{280}$ as a measure of purity (*see* **Note 3**).

## 3.2. General PCR Procedures

1. Prepare a master mix consisting of PCR buffer, nucleotides, primers, enzyme, and water. Depending on the precise assay, either 25 or 50 μL is allowed for each assay. To prepare 1 mL of our regular master mix, to a 1.5-mL microfuge tube add 100 μL of 10X PCR buffer; 100 μL of nucleotide stock; 10 μL each of primer 1 and primer 2, assuming a 25 μ*M* stock; 595 μL (if necessary, adjust the volume to ensure a final volume of 1 mL) of sterile water; and, finally, 25 U (usually 5 μL) of *Taq* polymerase. Perform all pipetting using automatic pipets reserved for this purpose (*see* **Note 4**). Mix gently and spin briefly in a microcentrifuge.
2. Pipet the appropriate volume (25 or 50 μL) into 0.5-mL microcentrifuge tubes. Add genomic DNA (100 ng to 1 μg) for amplification (*see* **Note 5**). Select at least one tube as a "no DNA" blank, and include appropriate controls of known genotype (*see* **Note 3**). Centrifuge briefly and overlay with 100 μL of mineral oil (*see* **Note 6**).
3. Place the tubes in a thermocycler programmed for the recommended temperatures and numbers of cycles (see individual assays for details).

## 3.3. Analysis of PCR Products by Electrophoresis

### 3.3.1. Use of Electrophoresis

On completion of the PCR reaction, it is often useful to determine whether the amplification has been successful using gel electrophoresis before additional analysis is performed. The precise type of gel and electrophoresis conditions will depend on several factors including the size of the fragments to be analyzed and whether separation of a number of fragments of similar molecular weight is required. It is essential that molecular weight standards be run on all gels. For most purposes, we use 100-bp DNA ladder markers, and in the protocols presented here, it is assumed that this is the recommended molecular weight marker unless otherwise stated.

### 3.3.2. Agarose Gel Electrophoresis

Agarose 7.6 × 10 cm minigels are run using a GNA-100 apparatus from Amersham Biosciences (Amersham, UK) and are prepared by dissolving the required weight of

agarose in 50-mL of TBE buffer in a conical flask. We typically run 2 or 3% gels to analyze digestion products or 1% to observe the results of a PCR amplification.

1. Dissolve agarose by heating in a microwave oven or on a Bunsen burner with regular mixing.
2. Allow the solution to cool to approx 60°C and add EtBr to a final concentration of 0.5 μg/mL.
3. Pour the solution into a gel mold, add two combs to form the sample wells, and allow the gel to set.
4. Transfer the gel to an electrophoresis tank and completely cover with TBE buffer.
5. Mix up to 8 μL of gel sample with 2 μL of gel-loading buffer and apply to the gel using an automatic pipet.
6. Carry out electrophoresis at 70 V for 30–60 min until the tracker dyes are well separated.
8. Visualize the DNA bands on a transilluminator.

### 3.3.3. Polyacrylamide Gel Electrophoresis

For analysis of PCR products by polyacrylamide gel electrophoresis, we run 15 × 17 cm gels using the Model V15.17 vertical gel electrophoresis system (Thistle Scientific, Glasgow, UK).

1. Prepare a 10% agarose gel by mixing 16.7 mL of 30% acrylamide solution and 5 mL of 10X TBE buffer and water up to 50 mL in a measuring cylinder. Add 0.5 mL of 10% ammonium persulfate and 50 μL of TEMED, and after rapid mixing, pour the gel solution between sealed gel plates and apply a well-forming comb to the top of the gel.
2. Allow the gel to polymerize for 30 min. Place the gel in an electrophoresis tank and add an appropriate volume of TBE. Mix up to 20 μL of sample with 5 μL of gel-loading buffer and apply to the wells using an automatic pipet.
3. Carry out electrophoresis at 150 V (constant voltage) until the bromophenol blue marker has reached the bottom of the gel (typically 4 to 5 h).
4. Stain the gel in 300 mL of EtBr solution (0.5 μg/mL) for 15 min, and after brief destaining in water, view on an ultraviolet (UV) transilluminator.

### 3.3.4. SSCP Gels

SSCP is performed with 15 × 17 cm gels using the Model V15.17 vertical gel electrophoresis system (*see* **Subheading 3.3.3.**).

1. Prepare an SSCP gel by dissolving 5 g of urea in 25 mL of Mutation Detection gel solution and 5 mL of 10X TME and then making up to 49.5 mL with water. Add 0.5 mL of 10% (w/v) ammonium persulfate and 50 μL of TEMED. After rapidly mixing, pour the gel solution between sealed gel plates and insert a well-forming comb in the top of the gel. This protocol will make sufficient gel solution for 1.5-mm-thick gel. Halve the quantities if a 0.7-mm-thick gel is to be poured. Be aware that thin gels are cheaper to use but that the sample volume that can be loaded on them is reduced, which may result in loading insufficient sample to be adequately visualized.
2. Allow the gel to polymerize for 30 min, and then place in an electrophoresis tank and add an appropriate volume of TME buffer.
3. Samples must be heat denatured before loading. Mix 8 μL of PCR product with 25 μL of SSCP loading buffer and incubate at 92°C for 3 min. Immediately place on ice and load the gel as soon as possible.

4. Perform electrophoresis at 150–200 V (constant voltage) for 12–24 h. The purpose of the xylene cyanol dye is to aid loading, and it will run off the bottom of the gel before electrophoresis has completed. The precise voltage, running time, and temperature at which to run the gel must be optimized for each PCR product.
5. Perform electrophoresis at either 4°C or room temperature (*see* **Note 7**).
6. Stain the gel in 200 mL of SYBR Gold solution in TBE buffer for 20 min, and visualize on a UV transilluminator.

### 3.4. Digestion of PCR Products With Restriction Enzymes

A number of the genotyping assays described in **Subheadings 3.5.–3.11.** involve restriction digests. These assays are normally carried out in PCR buffer (*see* **Note 2**) at the temperature recommended by the supplier. Typically either 10 or 20 µL of PCR product is digested with 1–10 U of enzyme.

### 3.5. CYP2D6 Genotyping

A large number of variant *CYP2D6* alleles have now been described ***(1,2,4)***. However, at least 95% of those homozygous or heterozygous for inactivating mutations can be detected by genotyping for the four alleles described in **Subheadings 3.5.1.–3.5.3.** (*see* **Note 8**).

#### 3.5.1. CYP2D6*3 *and* CYP2D6*4 *Alleles*

The forward primer is G1 (5'-TGCCGCCTTCGCCAACCACT-3') and the reverse is B1 (5'-GGCTGGGTCCCAGGTCATAC-3') (*see* **Note 9**). Each amplification is carried out in a volume of 25 µL with 0.2–0.5 µg of genomic DNA. After initial incubation for 1 min at 93°C, 35 cycles of incubation for 1 min at 93°C, 1.5 min at 63°C, and 5 min at 70°C followed by 1 cycle of 10 min at 70°C are carried out. A PCR product of 826 bp is produced by the reaction. Twenty microliters of product is digested with 1 µL of *Bst*NI (at 10 U/µL) and 1 µL of *Bsa*AI (at 5 U/µL) at 50°C for 3 h. To control for digestion by *Bsa*AI, 3 µL of a 50-µL PCR reaction of a region of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene can be added to the digest (*see* **Note 10**). The GAPDH gene product (591 bp) contains a single *Bsa*AI site and is digested to give two bands at 500 and 91 bp. A similar control for *Bst*NI digestion is not necessary, because there are several sites for the enzyme in the amplified CYP2D6 fragment, in addition to the polymorphic site.

The digestion products are analyzed on a 10% polyacrylamide gel (*see* **Subheading 3.1.3.3.**). The various band patterns observed with different genotypes are shown schematically in **Fig. 1**. All samples except those from subjects homozygous for the *CYP2D6*5* allele should contain invariant bands of 139, 63, 28, 25, and 10 bp. Subjects with at least one allele other than *CYP2D6*3* and *CYP2D6*4* should show additional bands of 280, 161, 100, and 20 bp. Subjects positive for *CYP2D6*4* will show additional bands of 380, 161, 100, and 20 bp. Subjects positive for *CYP2D6*3* will show additional bands of 280, 181, and 100 bp (*see* **Note 11**).

#### 3.5.2. CYP2D6*5 *Allele*

The forward primer is CYP-13 (5'-ACCGGGCACCTGTACTCCTCA-3') and the reverse is CYP-24 (5'-GCATGAGCTAAGGCACCCAGAC-3'). Each amplification

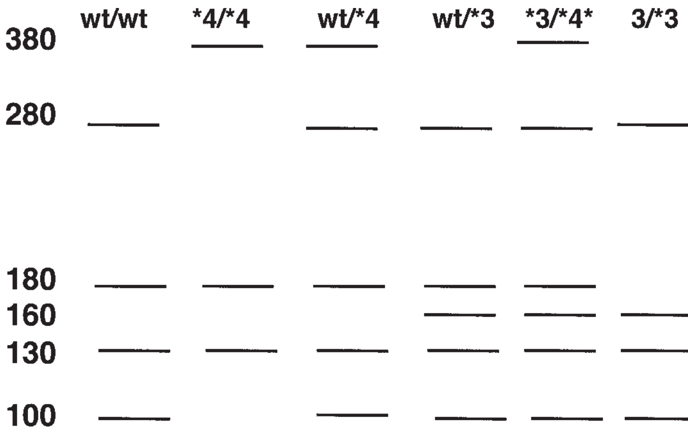


Fig. 1. Detection of *CYP2D6*3* and *CYP2D6*4* alleles by polymerase chain reaction (PCR). A schematic representation of a typical polyacrylamide gel revealing a range of band patterns is shown. The band sizes are shown in base pairs. wt, wild-type by this assay.

is performed in a total volume of 25 µL using enzyme and buffer 1 from the PCR-Expand system and 0.2–0.5 µg of genomic DNA. The PCR reaction conditions are 1 cycle of 1 min at 93°C followed by 30 cycles of 1 min at 93°C, 2 min at 65°C, and 10 min at 68°C followed by 1 cycle of 10 min at 70°C.

The PCR products are analyzed on a 1.0% agarose gel with *Hind*III-digested λ as the molecular weight standard. Samples positive for the *CYP2D6*5* allele (either heterozygous or homozygous) give a product of 3.5 kb. If both alleles are *CYP2D6*5* negative, no PCR product is produced (*see* **Note 12**). All samples are assayed in duplicate (*see* **Note 12**).

### *3.5.3.* CYP2D6*6 *Allele*

To prevent interference by *CYP2D* pseudogenes, a *CYP2D6*-specific long-fragment PCR must be performed first, and the *CYP2D6*6* assay is then performed on this product. The forward primer for the initial PCR is JM2 (5'-GTGTGTCCAGAGGAGCCCA-3') and the reverse is JM3 (5'-TGCTCAGCCTCAACGTACCC-3'). The PCR conditions are 35 cycles of 1 min at 93°C, 1 min at 62°C, and 5 min at 68°C. Each amplification is performed in a total volume of 25 µL using enzyme and buffer 3 of the Roche Expand PCR system and 0.2–0.5 µg of genomic DNA. A product of 4.2 kb is produced by this reaction.

A second PCR is performed using 1 µL of the long PCR as template. The forward primer is P3 (5'-CCTGGGCAAGAAGTCGCTGGACCAG-3') (*see* **Note 13**) and the reverse is G2 (5'-CTCGGTCTCTCGCTCCGCAC-3'). Each amplification is carried out in a volume of 30 µL using regular master mix. The PCR conditions are 35 cycles of 1 min at 93°C, 1 min at 61°C, and 1 min at 72°C. A product of 346 bp is produced by this reaction. Twenty microliters of the product is digested with 2 U of *Bst*NI at

60°C for at least 3 h and analyzed by electrophoresis through a 10% polyacrylamide gel. Subjects negative for both *CYP2D6*4* and *CYP2D6*6* will show bands of 183 bp and 161 bp. Subjects positive for *CYP2D6*4* will show a band of 344 bp. Subjects positive for *CYP2D6*6* will show bands of 183 and 141 bp as well as a 20-bp band, which is not usually detectable because it is so small. The same *CYP2D6*4* genotype should be obtained from this assay as from the combined *CYP2D6*3/CYP2D6*4* assay described in **Subheading 3.5.1.**

### 3.6. CYP2C19 Genotyping

Two PCR assays are carried out in parallel to detect the *CYP2C19*2* and *CYP2C19*3* alleles, respectively. Together these assays should allow identification of at least 80% of all individuals who have defective CYP2C19 activity (*see* **Note 14**).

#### 3.6.1. CYP2C19*2 *Allele*

The forward primer is MEP-1 (5'-ATTGAATGAAAACATCAGGATTG-3') and the reverse is MEP-2 (5'-GTAAGTCAGCTGCAGTGATTA-3'). Each amplification is carried out in a volume of 50 µL and 0.5–1.0 µg of genomic DNA is used. The PCR reaction conditions are 40 cycles of 1 min at 93°C, 1.75 min at 55°C, and 2 min at 70°C followed by 1 cycle of 10 min at 70°C. A PCR product of 169 bp is produced by the reaction.

Ten microliters of PCR product are incubated with 2 U of *Sma*I for 3 h at 37°C. The digestion products are analyzed on a 2% agarose gel. A homozygous wild-type (WT) sample will show bands at 120 and 49 bp. The 169-bp product remains undigested in a sample homozygous for *CYP2C19*2*. A heterozygous sample will show bands at 169, 120, and 49 bp.

#### 3.6.2. CYP2C19*3 *Allele*

The forward primer is MEP-32 (5'-TATTATCTGTTAACTAATATGA-3') and the reverse is MEP-4 (5'-ACTTCAGGGCTTGGTCAATA-3'). Each amplification is carried out in a volume of 50 µL with 0.5–1 µg of genomic DNA. The conditions are 30 cycles of 1 min at 93°C, 1.5 min at 52°C, and 2 min at 70°C. A product of 271 bp is obtained.

Twenty microliters of the product is digested with 15 U of *Bam*HI at 37°C for 3 h, and the digest is analyzed by electrophoresis on a 2% agarose gel. The presence of the *CYP2C19*3* allele is indicated by loss of the single *Bam*HI site. A homozygous WT sample will show bands at 175 and 96 bp. The 271-bp product remains undigested in a sample homozygous for *CYP2C19*2*. A heterozygous sample will show bands at 271, 175, and 96 bp.

### 3.7. CYP2E1

There are two well-characterized polymorphisms in the *CYP2E1* gene, which occur at –1019 bp upstream of the transcription start site (detectable with *Rsa*I) and at 7668 bp in intron 6 (detectable with *Dra*I), respectively ***(1)***. Under P450 nomenclature guidelines, alleles positive for both variant polymorphisms are denoted *CYP2E1*5A*, those positive for only the *Rsa*I variant are denoted **5B*, and those positive for only the *Dra*I variant are denoted **6* ***(4)***.

### *3.7.1.* RsaI *Polymorphism*

The forward primer is RSI (5'-TTCATTCTGTCTTCTAACTGG-3') and the reverse Ris S2 (5'-CCAGTCGAGTCTACATTGTCA-3'). Each amplification is carried out in a volume of 50 μL with 0.5–1 μg of genomic DNA. The PCR reaction conditions are 35 cycles of 1 min at 93°C, 2 min at 48°C, and 2 min at 70°C followed by 1 cycle of 10 min at 70°C. A PCR product of 410 bp is produced by the reaction.

Ten microliters of PCR product is incubated with 2 U of *Rsa*I for 3 h at 37°C. The digestion products are analyzed on a 2% agarose gel. A homozygous WT sample will show bands at 360 and 50 bp. The 410-bp product remains undigested in a homozygous mutant sample. A heterozygous sample will show bands at 410, 360, and 50 bp.

### *3.7.2.* DraI *Polymorphism*

The forward primer is DR3 (5'-TTCATTCTGTCTTCTAACTGG-3') and the reverse is DR4 (5'-CCAGTCGAGTCTACATTGTCA-3'). Each amplification is carried out in a volume of 50 μL using the regular master mix and 0.5–1.0 μg of genomic DNA. The PCR reaction conditions are 35 cycles of 1 min at 93°C, 2 min at 55°C, and 2 min at 70°C followed by 1 cycle of 10 min at 70°C. A PCR product of 995 bp is produced by the reaction.

Ten microliters of PCR product is incubated with 2 U of *Dra*I for 3 h at 37°C. The digestion products are analyzed on a 1.0% agarose gel. A homozygous WT sample will show bands at 572, 302, and 121 bp. A homozygous mutant sample will show bands at 874 and 121 bp. A heterozygous sample will show bands at 874, 572, 302, and 121 bp.

## *3.8. CYP2C9*

A number of different CYP2C9 polymorphisms are known. Three variant alleles, *CYP2C9*2* (codon 144), *CYP2C9*3* (codon 359), and *CYP2C9*11* (codon 335), result in amino acid substitutions that appear to affect enzyme activity and are seen at frequencies above 1% in Europeans ***(1,2,4)***.

### *3.8.1.* CYP2C9*2 *Allele ($R_{144}C$ Polymorphism)*

The forward primer is C9a (5'-GGATATGAAGCAGTGAAGGAA-3') and the reverse is C9b (5'-GGCCTTGGTTTTTCTCAACTC-3'). Each amplification is carried out in a volume of 30 μL using the regular master mix and 0.2–0.5 μg of genomic DNA. The PCR conditions are 35 cycles of 1 min at 93°C, 1.5 min at 60°C, and 2 min at 70°C. A PCR product of 420 bp is produced by the reaction.

Twenty microliters of the product are digested with 2 U of *Ava*II at 37°C for 3 h, and the digest is analyzed by electrophoresis on a 2% agarose gel. The presence of the mutation is indicated by loss of the single *Ava*II site. A homozygous mutant sample will show a single band at 420 bp. A homozygous WT sample will show bands at 363 and 57 bp. A heterozygous sample will show bands at 420, 363, and 57 bp.

### *3.8.2.* CYP2C9*3 *Allele ($I_{359}L$ Polymorphism)*

The forward primer is C9c (5'-TGCACGAGGTCCAGAGATGC-3') and the reverse is C9d (5'-AAACATGGAGTTGCAGTGTA-3'). The forward primer contains a single

base-pair mismatch that generates a new *Nsi*I site in individuals with the WT sequence present. Each amplification is carried out in a volume of 30 µL using the regular master mix and 0.2–0.5 µg of genomic DNA. The PCR conditions are 35 cycles of 1 min at 93°C, 2 min at 59°C, and 2 min at 70°C. A product of 131 bp is obtained.

Twenty microliters of the product are digested with 2 U of *Nsi*I for 3 h at 37°C, and the digest is analyzed by electrophoresis on a 10% polyacrylamide gel. The presence of the *CYP2C9*3* allele is indicated by the loss of the single *Nsi*I site created by the PCR primer. Samples from individuals homozygous for *CYP2C9*3* will give a single band of 131 bp, whereas those from heterozygous individuals will give bands of 131, 110, and 21 bp and those from individuals negative for *CYP2C9*3* will give bands of 110 and 21 bp.

#### *3.8.3.* CYP2C9*11 *Allele ($R_{335}W$ Polymorphism)*

The forward primer is IN6F (5'-CTGAATTGCTACAACAAATGTG-3') and the reverse is C9H (5'-AAACATGGAGTTGCAGTGTAG-3'). Each amplification is carried out in 25 µL of standard master mix and 0.2–0.5 µg of genomic DNA. The PCR conditions are 35 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 70°C followed by a single cycle of 7 min at 72°C. A product of 323 bp is obtained.

Five microliters of PCR product are added to a final reaction volume of 20 µL including 2 U of *Bsr*I and the recommended restriction buffer for this enzyme. The reaction is incubated at 65°C for 6 h. The digest is analyzed by electrophoresis on a 10% polyacrylamide gel. The presence of the *CYP2C9*11* allele is indicated by a restriction site for *Bsr*I. Samples from individuals homozygous for *CYP2C9*11* will give two bands of 188 and 135 bp, whereas those from heterozygous individuals will give three bands of 323, 188, and 135 bp and those from individuals negative for the allele will give a single band of 323 bp.

### *3.9. CYP2C8*

A number of different CYP2C8 polymorphisms are now known ***(4)***. Two variant alleles, *CYP2C8*3* (codons 139 and 399) and *CYP2C8*4* (codon 264), result in amino acid substitutions that may affect enzyme activity ***(5,6)***. Each occurs at frequencies above 1% in Europeans. *CYP2C8*3* includes two linked polymorphisms, but at present we recommend that both should be screened for (*see* **Note 15**), and methods are therefore described below for three separate genotyping assays. Two of these involve PCR followed by restriction digests, and the third involves PCR followed by SSCP analysis.

#### *3.9.1.* CYP2C8*3 *Allele*

##### 3.9.1.1. $R_{139}K$

The forward primer in the intron 2/exon 3 boundary region is N3.1 (5'-TTTTTATT AGGAATCATTTC-3') and the reverse primer, in the exon 3/intron 3 boundary region is N3.2 (5'-AGTCACCCACCCTTGGTTTT-3'). Each amplification is carried out in a volume of 30 µL using regular master mix and 0.2–0.5 µg of genomic DNA. The PCR conditions are 35 cycles of 1 min at 93°C, 1 min at 48°C, and 1 min at 72°C. A PCR product of 170 bp is produced by this reaction.

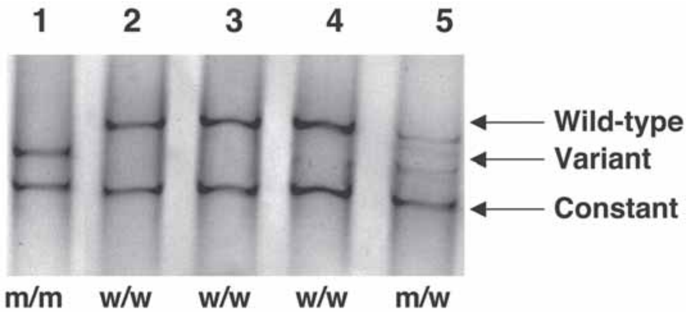


Fig. 2. Genotyping for codon 399 polymorphism (R399K) of *CYP2C8*3* allele by SSCP analysis: lane 1, a homozygous mutant (m/m), lanes 2–4, samples negative for the polymorphism (w/w); lane 5, a heterozygote (m/w).

Fifteen microliters of the product are digested with 2 U of *Bse*RI in a total volume of 30 µL consisting of 15 µL of PCR product and 15 µL of 1X *Bse*RI buffer at 37°C for 16 h. The digest is resolved by electrophoresis on a 10% acrylamide gel. A homozygous WT sample will show three bands of 110, 40, and 20 bp. The 20-bp band may be too faint to visualize. A homozygous mutant sample will show two bands of 150 and 20 bp and a heterozygote will show bands of 150, 110, 40, and 20 bp.

#### 3.9.1.2. $K_{399}R$

The forward and reverse primers are in the intron sequence on either side of exon 8. The forward primer is Ex8F (5'-ACTACTTCTCCTCACTTCTG-3') and the reverse is Ex8R (5'-TGCCATGTAAATTCCAACTA-3'). Each amplification is carried out in a volume of 25 µL using regular master mix and 0.2–0.5 µg of genomic DNA. The PCR conditions are 35 cycles of 1 min at 93°C, 1 min at 56°C, and 1 min at 72°C. A PCR product of 277 bp is produced by this reaction.

Eight microliters of product is analyzed by SSCP. The gel is run at 200 V (constant voltage) at 4°C for 20 h. A homozygous WT sample will show two bands with good separation. A homozygous mutant sample will also show two bands, the lower band in the same position as the WT, but the upper band has noticeably greater mobility and will be closer to the lower band compared with the WT (*see* **Fig. 2**).

### *3.9.2.* CYP2C8*4 *($I_{264}M$)*

The forward primer in exon 5 is C8.1(5'-AAAAATGTTGCTCTTACACG-3') and the reverse primer in the exon 5/intron 6 boundary region is C8.2 (5'-ATTTTACCT GCTCCATTTTG-3'). Each amplification is carried out in a volume of 25 µL using regular master mix and 0.5 µg of genomic DNA. The PCR conditions are 35 cycles of 1 min at 93°C, 1 min at 54°C, and 1 min at 72°C. A PCR product of 125 bp is produced by this reaction.

Fifteen microliters of the product are digested with 2 U of *Taq*1 at 65°C for 16 h, and the digest is resolved by electrophoresis on a 3% agarose gel. A homozygous WT

sample will show two bands of 90 and 35 bp. The 35-bp band may be too faint to visualize. A homozygous mutant sample will not be digested.

### 3.10. CYP3A5

A polymorphism in CYP3A5, $A_{6986}G$, has been demonstrated to be associated with lack of expression *(7)*. The variant allele, *CYP3A5*3*, leads to the insertion of an intron sequence into the mature mRNA sequence and the production of a truncated protein.

#### *3.10.1.* CYP3A5*3 *Allele*

The forward primer is 3A5A (5'-CCTGCCTTCAATTTTCACT-3') and the reverse, 3A5B (5'-GGTCCAAACAGGGAAGAGGT-3') (*see* **Note 16**). Each amplification is performed in a volume of 25 µL using standard master-mix and 0.2–0.5 µg of genomic DNA. The PCR conditions are 35 cycles of 1 min at 94°C, 1 min at 61°C, 1 min at 70°C, and a single cycle of 7 min at 72°C. A product of 196 bp is amplified by this reaction.

Twenty microliters of the PCR product is incubated with 3 U of the enzyme *Rsa*I overnight at 37°C. The digest is analyzed on a 10% polyacrylamide gel. A naturally occurring *Rsa*I site in the PCR product leads to the digestion of all samples into fragments of sizes 102 and 94 bp. The engineered *Rsa*I site in the sequence of those individuals positive for G at position 6986 (*CYP3A5*3*) gives a further digestion of the 94-bp fragment into two fragments of 74 and 20 bp. Samples homozygous for *CYP3A5*3* will give bands of 102, 74, and 20 bp, and heterozygous samples will give bands of 102, 94, 74, and 20 bp. Samples negative for *CYP3A5*3* will give two fragments of 102 and 94 bp.

### 3.11. CYP1A1

Two polymorphisms, both resulting in amino acid substitutions, are known to occur in exon 7 of CYP1A1. The variant alleles have been termed *CYP1A1*2B* and *CYP1A1*4* and result in amino acid substitutions of $Ile_{462}Val$ and $Thr_{461}Asn$, respectively. The assay used for detection of *CYP1A1*2B* also detects *CYP1A1*4,* and to differentiate between these two alleles, it is necessary to carry out a second PCR assay that specifically detects *CYP1A1*4.*

The forward primer is 1A1X (5'-GAACTGCCACTTCAGCTGTCT-3') and the reverse is 1A1Y (5'-CCAGGAAGAGAAAGACCTCCCAGCGGGCCA-3') (*see* **Note 17**). Each amplification is carried out in a volume of 50 µL using the regular master mix and 0.5 µg of genomic DNA. PCR conditions are 35 cycles of 1 min at 94°C, 1 min at 68°C, and 1 min at 70°C. A PCR product of 195 bp is obtained.

The product is digested with *Nco*I and the digests are analyzed by electrophoresis on 2% agarose gels. The presence of either the *CYP1A1*2B* or *CYP1A1*4* allele is indicated by a single band of 195 bp owing to loss of the single *Nco*I site. For other alleles, two bands of 163 and 32 bp are obtained.

For subjects who are either homozygous or heterozygous for *CYP1A1*2B/CYP1A1*4*, an additional assay that discriminates between *CYP1A1*2B* and *CYP1A1*4* is carried out. The forward primer is 1A17FP (5'-CCACTCACTTGACACTTCTG) and the reverse is 1A17RP (5'-TAGACAGAGTCTAGGCCTCA). Each amplification

is carried out in a volume of 50 μL using the regular master mix and 0.5–1.0 μg of genomic DNA. The PCR conditions are 35 cycles of 1 min at 93°C, 1.5 min at 55°C, and 2 min at 70°C. A product of 381 bp is obtained.

Twenty microliters of the product are digested with *Bsa*I for 5 h at 50°C, and the digests are analyzed by electrophoresis on 1.8% agarose gels. The presence of the *CYP1A1*4* allele is indicated by the absence of a *Bsa*I site and a single band of 381 bp. The presence of a *Bsa*I site that gives two bands of 201 and 180 bp indicates that the positive result with *Nco*I is owing to the presence of the *CYP1A1*2B* allele (*see* **Note 17**).

## 4. Notes

1. A variety of thermostable DNA polymerases are available. For many of the assays, *Taq* DNA polymerase from a variety of suppliers will give satisfactory results. For amplifications of templates in excess of 2 kb, we recommend the use of a kit intended for long-range PCR. These kits are available from several suppliers and generally consist of a mixture of a DNA polymerase without a proofreading 3' to 5' exonuclease activity, such as *Taq* DNA polymerase, and a proofreading enzyme, such as *Pwo* DNA polymerase. For additional information on thermostable polymerases *see* **ref. *8***.
2. Rather than the KCl buffer described here, a number of enzyme suppliers' buffers contain ammonium ions. Although there may be some advantages in using these ammonium-containing buffers for PCR, they are not suitable for use in restriction digests. With the KCl buffer, the majority of enzymes show sufficient activity to allow direct digestion of PCR products following amplification, and we therefore recommend using this when possible. Further information is provided in **ref. *8*.** As an alternative, if a high yield of PCR product is obtained, 2–5 μL of product can be used in a 20-μL final volume restriction digest to which the normal restriction enzyme buffer is also added.
3. Yield and purity of genomic DNA are determined by measuring the absorbance at 260 nm and 280 nm. On the basis of the $A_{260}$ reading, DNA concentration is calculated with an absorbance of 1 equivalent to a DNA concentration of 50 μg/mL. If the $A_{260/280}$ ratio is <1.6, the sample may be contaminated with protein, resulting in difficulties with amplification by PCR. Further purification may be necessary. If only small quantities of starting material are available, it may not be possible to quantitate the amount of DNA present but it is still possible to obtain a PCR product. Information on obtaining DNA from archival samples such as pathological specimens is provided in **ref. *9*.**
4. PCR is a sensitive technique, and there is a risk that incorrect results may be obtained owing to contamination of samples with small amounts of PCR product from previous amplifications. The risk of this occurring can be greatly reduced by using separate areas of the laboratory for DNA preparation, for setting up PCR reactions, and for analyzing PCR products. It is particularly important that different pipets be used for each of these processes. It is also important that controls for contamination detection be included in each set of assays. These should include a "no DNA" blank tube with all other components present and at least one sample of known genotype. It is also valuable to genotype samples on two separate occasions. More information on avoiding contamination and decontamination methods is provided in **ref. *8***.
5. We routinely add 0.2–0.5 μg of DNA to a 25- or 50-μL reaction but have obtained PCR products from 100 ng of starting material when necessary.

6. To avoid evaporation of the reaction, it is essential that it be overlaid with light mineral oil. This is not detrimental to the reaction or to subsequent sample processing. Alternatively, some thermocyclers have heated lids to prevent evaporation.
7. When using SSCP for the detection of novel polymorphisms, it is usual to run gels in parallel at two different temperatures and/or use slightly different gel compositions to achieve maximum sensitivity for detection ***(10)***. For more routine genotyping, the single temperature giving the best separation, which is most commonly 4°C, is used.
8. At least 40 different defective CYP2D6 alleles have now been described ***(4)***. However, current evidence ***(1)*** suggests that for most purposes, genotyping for the four most common defective alleles (*CYP2D6*3, CYP2D6*4, CYP2D6*5,* and *CYP2D6*6*) will identify in excess of 95% of poor metabolizers. The other alleles tend to be extremely rare, and assaying for them will not greatly improve sensitivity of detection of poor metabolizer.
9. The reverse primer has a single base-pair mismatch that results in a *Bsa*AI site being generated if the $A_{2637}$ deletion characteristic of the *CYP2D6*3* allele is also present.
10. Including a positive control for *Bsa*AI digestion can be useful. *Bst*NI digestion is already internally controlled owing to the presence of several invariant sites in the PCR product. For *Bsa*AI, we add a GAPDH PCR product that contains a *Bsa*AI site to the digestion. The product is prepared using the forward primer R4 (5'-AGAAACAGGAGGTCCCTACT-3') and the reverse primer R5 (5'-GTCGGGTCAACGCTAGGCTG-3') and 35 cycles of 1 min at 93°C, 1.5 min at 58°C, and 2 min at 70°C followed by 1 cycle of 10 min at 70°C. A PCR product of 591 bp is produced by the reaction and yields digestion products of 500 and 91 bp when digested with *Bsa*AI.
11. Poor metabolizers of CYP2D6 will have two defective alleles. Genotype should be assigned on the basis of the data from the three assays described in **Subheadings 3.1.5.–3.5.3.** Individuals who fail to give a product for both the *CYP2D6*3/*4* and the *CYP2D6*6* assays but are positive for *CYP2D6*5* are *CYP2D6*5* homozygotes. No linkage had been observed among the inactivating mutations characteristic of *CYP2D6*3*, **4,* and **6* ***(4)*** and, therefore, subjects who are heterozygous for two of these alleles are assumed to have two defective alleles and to be poor metabolizers.
12. Since no PCR product is obtained in individuals lacking the *CYP2D6*5* allele, an internal positive control would be useful. Attempts to design such a control have not yet been successful, and it is therefore suggested that the *CYP2D6*5* assay be carried out in duplicate on two separate occasions and that positive and negative controls should always be included.
13. The forward primer has a single base-pair mismatch that results in a *Bst*NI site being generated if the T deletion characteristic of the *CYP2D6*6* allele is also present.
14. The *CYP2C19*3* allele is very uncommon among Europeans but occurs in approx 20% of Oriental subjects lacking CYP2C19 activity.
15. Although the codon 139 and 399 polymorphisms have been shown to be in complete linkage disequilibrium in two separate studies ***(5,6)***, we have more recently found rare cases of individuals positive for only one of these polymorphisms. The functional significance of being positive for a single polymorphism remains unclear, but screening for only one of the polymorphisms may result in misleading predictions.
16. The reverse primer contains a single base-pair mismatch that creates an *Rsa*I site in individuals with G at position 6986 and who therefore are heterozygous or homozygous for the *CYP3A5*3* allele.

17. The reverse primer contains a single base-pair mismatch that introduces a *Nco*I site into the WT sequence ***(11)***.

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# 25

# Hepatocyte Cultures in Drug Metabolism and Toxicological Research and Testing

**Tamara Vanhaecke and Vera Rogiers**

**Summary**

When a new chemical entity is tested for its safety for humans and their environment, in vivo experiments on living animals are usually conducted. However, in the early preclinical stage of drug development, in vitro techniques, and more specifically hepatocyte-based in vitro models, are currently being applied. A major problem, however, related to the use of hepatocytes and their cultures is their limited viability, which is associated with the loss of phase I and phase II biotransformation capacity. Therefore, in order to keep the hepatocytes functional for a longer period, the in vivo microenvironment is mimicked in vitro as closely as possible through the addition of soluble medium components, coculture with helper cells, or culture on an extracellular matrix (sandwich culture). We discuss the advantages and disadvantages of current short- and long-term hepatocyte culture systems are discussed as well as their proper use in toxicological research and testing.

**Key Words:** Short-term primary hepatocyte cultures; long-term primary hepatocyte cultures; metabolic competence; toxicity testing.

## 1. General Background on the Use of Hepatocyte-Based In Vitro Models

The safety and efficacy of new pharmaceuticals for human use is ensured by in vivo testing, at a first stage by using experimental animals and, later, in clinical trials by administering of the drugs to human volunteers *(1–3)*. On a global scale, animal-based in vivo testing involves a large number of vertebrates, particularly rodents *(4)*.

As the safety criteria of new drugs progressively increase, so does the use of experimental animals. However, this is no longer ethically, economically, or scientifically acceptable. The solution to this problem may lie, at least in part, in the consistent use of in vitro models in drug metabolism and toxicological research and testing.

The liver is the major organ with respect to drug metabolism. Because drug metabolism and toxicity are inherently linked and the liver is known to be one of the most commonly affected target organs in preclinical toxicity studies, several liver-based in

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vitro models have been developed, including purified enzymes, subcellular fractions, isolated hepatocytes in suspension and in culture, immortalized liver cell lines, recombinant liver cell lines, liver slices, and even perfused whole organs ***(5,6)***.

Major problems are associated with the use of such liver-based in vitro models, owing to either limited viability or the reduction or loss of important differentiated liver-specific functions ***(2,6–8)***. Consequently, the practical applicability of isolated hepatocytes in suspension (4–5 h) and in short-term primary monolayer cultures (2–3 d) is limited. Nevertheless, hepatocytes have been successfully used to investigate drug metabolism, cytotoxicity, hepatotoxicity, and genotoxicity, as well as for mechanistic studies of biological and/or toxicological action, biokinetic studies, and species selection ***(2,9,10)***. Some toxicological problems, however, can be addressed only by longer exposure of cells to the drugs under investigation. These problems include inducing capacities of new molecules on xenobiotic biotransformation, some drug interactions, long-term effects of endogenous and exogenous factors on drug metabolism patterns, and subchronic/chronic toxicity of xenobiotics ***(11–13)***. Consequently, the development, characterization, and validation of long-term cultures of hepatocytes as in vitro experimental models represent an important goal in experimental and regulatory toxicology, drug development, and risk assessment. To be of value for drug development, such models should be able to express key phase I and phase II drug-metabolizing capacities comparable with those in vivo and for longer time periods (2–3 wk) than is possible with short-term conventional hepatocyte monolayer cultures. At present, such an ideal culture system does not exist, although it is clear that the more sophisticated hepatocyte culture systems display promising properties ***(6,12–14)***. These models take into account a number of elements of the microenvironment including cell-medium/matrix interactions, medium/matrix components, cell–cell interactions, cell shape, and polarity ***(13–15)***. Recently, attention has been more focused on reducing dedifferentiation of cultured hepatocytes through intervention at the molecular, rather than the cellular, level. Indeed, the addition of specific regulating molecules, such as the histone deacetylase inhibitor Trichostatin A, to the culture medium of hepatocytes or even during the isolation procedure of hepatocytes opens promising perspectives with respect to long-term maintenance of biotransformation capacity ***(16,17)***.

## 2. Metabolic Competence of Short-Term vs Long-Term Hepatocyte Cultures

### *2.1. Short-Term Cultures*

Short-term cultures or conventional monolayer cultures of hepatocytes are kept for 2 to 3 d, and during that limited period they maintain both phase I and phase II drug-metabolizing enzymes at an acceptable level in comparison to in vivo ***(3,18)***. Thus, they are reasonably effective models for establishing the biotransformation pattern of new drugs in different species, including human ***(2,9,19)***. For this reason, they have been widely used as an in vitro model in drug metabolism studies and for toxicological research and testing. They provide a good model for studying the direct effects of hepatotoxins on the cells and the toxic events caused by active metabolites formed.

As a function of culture time, however, hepatocytes undergo both morphological (flattening and spreading out) and phenotypic (acquisition of fetal characteristics, expression of immediate-early protooncognes *c-fos* and *c-myc*) changes. Consequently, after 3 d, or even earlier, they no longer accurately reflect xenobiotic biotransformation in vivo ***(12,19)***. Of particular concern is the reduction in the expression of several cytochromes P450 and the loss of response to some well-known inducers of these enzymes ***(2,20–22)***. A similar problem with respect to phase II drug-metabolizing enzymes ***(23–27)*** and non-cytochrome P450-dependent phase I metabolism ***(28)*** occurs. The observed decrease in biotransformation capacity in conventionally cultured hepatocytes is accompanied by an increase in apoptosis ***(29)***.

Hormonal responsiveness also seems to be limited in monolayer cultures of hepatocytes; whereas uridine diphosphate (UDP)-glucuronosyltransferase (UGT) isoenzyme mRNAs are affected by growth hormone, testosterone, dexamethasone, and triiodothyronine ($T_3$), the latter does not alter the glutathione-*S*-transferase (GST) activity as observed in vivo, even when high amounts of pyruvate are present ***(30,31)***.

High oxygen concentrations have also been shown to improve the metabolic functionality of conventionally cultured hepatocytes ***(32)*** and are important when studying the zonated expression of GST proteins ***(33)***.

### 2.2. Long-Term Cultures

From the literature, it is clear that the maintenance of the differentiated state of cultured hepatocytes as a function of time is strongly dependent on a complex environment in which exogenous factors, cell–matrix and cell–cell interactions play key roles ***(11–13,15)***. The more sophisticated culture models, including cocultures of hepatocytes with helper cells, collagen gel cultures, and liver spheroids, are thus better candidates to study long-term effects of drugs.

#### 2.2.1. *Cocultures of Hepatocytes With Helper Cells* (see *Chapter 27*)

Hepatocytes are cultured with nonparenchymal cells in order to mimic better the in vivo situation in which hepatocytes are in contact with nonparenchymal cells via the space of Disse.

Several continuous cell lines from different tissues have been used as helper cells ***(6,19)***, but the most successful coculture system was obtained with rat liver epithelial cells (RLECs) of primitive biliary origin ***(34)***. The hepatocytes not only display a longer viability but also retain to a greater extent the morphological and biochemical characteristics of parenchymal liver cells in vivo ***(12)***. This is true for hepatocytes from various species, including human, and from adults and fetuses ***(35–37)***. Cocultured rat hepatocytes reveal a steady-state situation for at least 10 d in which phase II enzymes are qualitatively maintained ***(23,24,26,38–40)***, the cytochrome P450 content is partly preserved ***(20)***, cytochrome P450 forms are selectively maintained ***(20,38,39,41)***, and the non-cytochrome P450-dependent flavin-containing monooxygenase (FMO) is well expressed ***(28)***. It could also be shown that the hormonal regulation of GST ***(42,43)*** and FMO ***(44)*** is preserved throughout the culture time, which is not the case in conventionally cultured hepatocytes ***(28)***. Furthermore, besides

being able to maintain the predominant perivenous expression of GST proteins for 4 d, the coculture system has been shown to mimic the $T_3$-mediated periportal suppression of GST α isoenzymes observed in vivo *(33)*.

The mRNAs encoding members of the CYP2B subfamily, CYP2B1 and CYP2B2, are maintained in cocultured rat hepatocytes, and they remain inducible by phenobarbital and sodium valproate (VP) to an extent comparable with that observed in vivo *(22,45,46)*. Also quantitatively expressed are the genes coding for nicotinamide adenine dinucleotide phosphate cytochrome P450 reductase, cytochrome-$b_5$,and P450 4A *(21,22)*.

The early production and deposition of extracellular matrix (ECM) components *(47,48)* has been suggested to play a key role in the maintenance of both high levels of gene transcription *(49)* and cell–cell communication via gap junctions *(50)*. It has also been shown that plasma–membrane constituents of RLECs, used in cocultures, are responsible for the stabilization of gap junctional intercellular communication between parenchymal cells *(51)*, and of a liver regulatory protein (LRP). The latter is present on both hepatocytes and epithelial cell membranes *(53)*. LRP has been shown to be involved in the transcription of liver-specific genes and/or transcription factors that regulate the differentiated phenotype of hepatocytes *(52,53)*. In addition, LRP participates in the control of ECM deposition and influences cytoskeletal organization *(52,53)*.

Coculture conditions have also been associated with less oxidative stress than observed in conventional culture conditions *(54,55)*. In addition, cocultured rat hepatocytes can deal better with oxidative stress from exogenous molecules than conventionally cultured cells *(56)*. The level of spontaneous apoptosis is also very low in cocultures compared to monolayer cultures of hepatocytes *(29)*, indicating a regulating role of cell–cell contacts in the process of cell death.

Perifusion coculture systems, with flow-through of the medium, seem to have an additional beneficial effect on both the viability of the hepatocytes and induction of their biotransformation enzymes *(57,58)*. Although these models could be promising for chronic toxicity testing, they are quite expensive and even more complicated than classic cocultures *(19)*.

Disadvantages of the coculture technique are related to the fact that isolation of the biliary epithelial cells is time-consuming and not well standardized. Furthermore, these cells are not well characterized and contribute to the DNA and protein contents, which are both used to normalize most biochemical and metabolic parameters.

#### *2.2.2. Collagen Gel Cultures* (see *Chapter 28*)

Two models of collagen gel cultures seem to be of particular importance: collagen gel sandwich configuration cultures and immobilization gel cultures of hepatocytes. In the former model, hepatocytes are sandwiched between two layers of rat tail tendon collagen type I *(59–61)* (or Matrigel *[62]*). In a first step, hepatocytes are seeded onto a collagen layer and allowed to attach. A second layer of collagen is then spread over the attached hepatocytes 1 d after seeding. The second layer of collagen prevents flat-

tening and spreading of the hepatocytes. A simple variation of this model is the collagen gel immobilization culture of hepatocytes. The cells are allowed to settle within a fluid collagen preparation before gelatination, which results in the entrapment of the cells within the gel matrix ***(63)***. In both systems, the cells retain their polarity and cuboidal shape, and an architecture similar to that found in vivo is retained ***(64–67)***. A distribution of actin filaments, resembling that present in vivo, is maintained throughout the entire culture period, which is in sharp contrast to the stress fibers that appear in the cytoplasm of conventionally cultured hepatocytes ***(60,68)***. Liver-specific functions, including the secretion of urea, albumin, transferrin, fibrinogen, and bile salts, are maintained for up to 6 wk ***(59,60,62,64,69,70)***. It has been claimed that continued synthesis of collagen by hepatocytes in culture may be critical for their function ***(71)***. Compared to conventional monolayer cultures, the level of spontaneous apoptosis in collagen gel sandwich cultures is diminished owing to a reduction in caspase-3 activation ***(29,72)***.

Relatively stable expression of CYP1A1, 1A2, 2A2, 2B1, 3A1, and 3B1 can be shown in rat hepatocyte sandwich cultures, whereas CYP2C11, 2A1, and 2B2 activities decrease as a function of culture time ***(73–77)***. In human hepatocyte sandwich cultures, CYP1A2 and 3A4 can be maintained for more than 1 wk ***(73,74,78,79)***. In addition, CYP1A1/2, 2A1/2, 3A1, 2B1/2, 2C11, and 2E1 are enhanced by prototypical inducers ***(76–78,80)***. Furthermore, the activity of the non-cytochrome P450-dependent phase I enzyme epoxide hydrase is stably expressed in both sandwich and immobilization gel cultures ***(76)***.

Phase II $\alpha$, $\mu$, and $\sigma$ class GST isoenzymes are stably expressed for at least 3 wk in both collagen gel culture models ***(81,82)***, but changes in the subunit pattern occur ***(83)***. The inducibility of GSTs by several inducers is well maintained ***(84)***. The expression of phase II sulfotransferase isoenzyme SULT1A1 is maintained at 70% of the in vivo level after 4 d of collagen gel culture, whereas SULT1C, 1E, 20/21, 40/41, and 60 expression are gradually lost ***(85,86)***. UGT-dependent activity is relatively well maintained ***(82)*** .

In human hepatocyte immobilization gel cultures, the CYP1A1-dependent *O*-demethylation of *p*-nitroanisole ***(63)*** and the *O*-demethylation and subsequent *O*-glucuronidation pathway of pimobendan could be maintained for 3 wk ***(87)***. Likewise, the conjugation patterns for acetaminophen were stable for 2 wk ***(63)***.

Practical constraints of both culture models are associated with the presence of collagen. To gain access to the hepatocytes for metabolic and toxicity analyses, the collagen usually has to be enzymatically digested ***(81)***. In addition, drugs or substrates added must be able to penetrate the collagen layer in order to reach the cells. Consequently, the physicochemical properties of the drugs are of importance ***(63)***. Another problem, which occurs particularly in immobilization gels, is the presence of dead and dying cells, which become entrapped during initiation of the cultures. They cannot be removed, as in conventional cultures and cocultures. Thus, leaking enzymes can damage neighboring cells and their protein and DNA contents can cause erroneous results in normalization of biochemical and metabolic parameters ***(81)***.

#### 2.2.3. Liver Spheroids

In the liver spheroid model, the attachment of hepatocytes to a solid support is inhibited, and they aggregate and form floating three-dimensional structures. One method is based on the culture of hepatocytes on nontissue-culture plastic and on a rotary shaker *(88)*. Other methods involve the culture of hepatocytes with nonadherent substrata, including poly (2-hydroxy- ethylmethacrylate) (p.HEMA), liver-derived proteoglycans, or a collagen-conjugated thermoresponsive polymer *(89–91)*. A combination of both the rotary shaker and coating with p.HEMA results in a faster and more efficient technique *(92)*.

In spheroids, hepatocytes maintain not only in vivo morphological characteristics, but also liver-specific functions, including the secretion of albumin, transferrin, and urea and the formation of bile acid for several weeks of culture *(93,94)*. Although loss of CYP2A1, 2E1, 2C6, and 2C11 is observed, CYP-dependent enzyme expression is relatively well maintained *(95–97)* and remains inducible by prototypical CYP inducers *(15,96–100)*. With respect to phase II drug metabolism, it is known that the expression of α and μ GST isoenzymes is maintained in spheroids, whereas GST π is increased *(15,101)*. In addition, normal lidocaine metabolism and peroxisomal proliferation, associated with hepatocarcinogenicity in rodents, have been reported *(100)*. Responsiveness to $T_3$ is also observed in hepatocyte spheroids *(102)*.

A disadvantage of the use of spheroids, however, is that larger sizes (200-μm diameter and greater) contain inner cells that degenerate because of dehydration. A diffusion gradient of nutrients, oxygen, as well as test compounds across the spheroids will limit the exposure of the inner cells *(103)*. Furthermore, functionality of liver spheroids strongly depends on culture conditions, in particular on the type of medium used *(14)*.

## 3. Toxicity Studies With Hepatocyte Cultures

### *3.1. Screening and Ranking of Toxic Compounds*

As already mentioned, freshly isolated hepatocyte suspensions and short-term cultures of hepatocytes represent the most popular in vitro models for comparing and ranking the relative toxicity of different classes of chemicals *(10,14,18)*. Ranking is done by determining the $IC_{50}$ value of each compound, which is the concentration of the test compound causing a 50% inhibition of the toxicity parameter measured. Toxicity parameters can be subdivided either into liver- and non-liver-specific toxicity markers or into basic cytotoxicity and metabolic competence markers (**Table 1**). However, a serious drawback of this method is the wide range of concentrations to be tested, with maxima far in excess of real plasma and tissue levels. Reasons for the wide range of concentrations could be either a low metabolic rate or a short exposure time in vitro. Thus, models allowing in vivo exposure times and metabolic rates (long-term culture models of hepatocytes) seem more promising for future work *(11,104)*.

The quality of the results obtained will depend not only on the choice of the culture model but also on the following:

1. The toxicity marker(s) chosen.
2. The in vitro biotransformation pattern and metabolic rate of the compound(s) under investigation (additional factors include age, sex, and species of cell source).

**Table 1**
**Liver-Specific and Nonliver-Specific Criteria as Quantitative Indicators of Toxicity *(12,101,116)***

| Liver-specific criteria | Nonliver-specific criteria |
|---|---|
| Specific plasma protein synthesis: albumin, transferrine | Plasma membrane integrity |
| Ureogenesis | Dye uptake (e.g., Trypan Blue) |
| Apolipoprotein synthesis | Intracellular enzyme leakage (e.g., lactate dehydrogenase) |
| Peroxisome proliferation | Intracellular ion leakage (e.g., $K^+$ release) |
| Cytochrome P450 induction/inhibition | Intracellular accumulation of external molecules (e.g., succi |
| Bile acid uptake, conjugation, secretion | Lysosomal integrity |
| Gluconeogenesis | Neutral red uptake |
| Glycogen synthesis | Levels of acid phosphatase |
| | Mitochondrial activity |
| | Adenosine triphosphate content/nucleotide ratios |
| | Oxygen consumption |
| | Reduction of a formazan salt (MTT test) |
| | DNA damage |
| | Metabolic competence |
| | Total protein synthesis |
| | Glutathione content |
| | Lipid peroxidation |
| | Covalent binding to macromolecules |

3. Standardization and validation of the protocols used (e.g., media composition, culture atmosphere, and substrates added).
4. The physicochemical properties of the compound(s) under study (e.g., volatility and lipid solubility).
5. The availability of in vivo data on the compound(s) tested (e.g., histological, biochemical, and toxicological).

Generally, the measurement of metabolic competence parameters is a more sensitive method than the use of basic cytotoxicity markers *(105,106)*. A typical example from our own research is the finding of a concentration-dependent inhibition of gluconeogenesis in rat hepatocyte suspensions caused by exposure to the antiepileptic compound sodium VP and its unsaturated 4-ene metabolite, and the lack of measurable lactate dehydrogenase leakage into the incubation medium *(107)*. Exposure of rat hepatocyte cultures to milacemide, another antiepileptic molecule, caused a significant inhibition of several phase I drug-metabolizing enzymes and also important morphological changes, whereas the lactate dehydrogenase leakage index again remained unchanged *(108)*.

However, there is some evidence that markers of metabolic competence are not always more sensitive than basic cytotoxicity end points in detecting the toxicity of a compound *(14)*. It should also be mentioned that the suitability of hepatocyte-based in vitro systems as models for screening compounds early in drug development is more convincing when the compounds to be ranked are structurally related.

Next to classic toxicity marker assays, genomic, proteomic, and metabolomic approaches have become quite popular in the screening of toxicants. As such, thousands of genes, proteins, and metabolites can be studied at the same time *(6,109)*. However, these recently developed techniques are rather expensive, limiting their use to pharmaceutical industries.

### 3.2. Studies of Genotoxic Compounds

Short-term rat hepatocyte cultures have been used for some years to help assess the genotoxic potential of chemicals and drugs *(110–113)*, because (1) they contain a broad spectrum of biotransformation enzymes, ensuring adequate activation of most xenobiotics; (2) they allow continuous treatments for several days, whereas in classic experiments with dividing cells the S9-mix restricts the treatment period to 2–6 h; and (3) no transfer of the activated metabolites is necessary *(114)*. As such, they are well suited for quantification of DNA fragmentation and DNA repair after exposure to genotoxic substances. These assays are considered valid models for predicting potential genotoxic effects in humans *(112–117)*. However, for some compounds, differences in response between rodent and human cells were noticed, highlighting again the importance of species differences *(118,119)*. These differences are sometimes quite subtle *(120)*. It is therefore suggested that whenever information on a human carcinogenic hazard must be obtained, human hepatocyte cultures will give more useful information provided that gender and interindividual differences are also taken into account *(119)*.

### 3.3. Mechanistic Studies

Hepatocyte suspensions and short-term cultures, particularly in combination with genomic and proteomic technology, are, again, the most popular in vitro models to

obtain valuable information on the nature of the hepatotoxic mechanism of a compound ***(6,10,109)***. Mechanisms to be studied may be, e.g., a solvent effect on the cellular membrane, affecting its physicochemical properties; the production of reactive oxygen species and free radicals, provoking lipid peroxidation of cellular membranes or glutathione depletion; and covalent binding to macromolecules ***(121)***. In such assays hepatocytes of different species, including human, may be used. Examples of successes achieved through the use of short-term cultures of human hepatocytes are the elucidation of the direct potentiation of the hepatotoxic effect of opioids by ethanol ***(122)*** and the mechanism of the hepatotoxic effect of acetaminophen ***(123)*** and diclofenac ***(124)***.

When the biotransformation of the compound under investigation is a key factor and long-term exposure is necessary to induce hepatotoxicity, long-term cultures may represent a far better in vitro model. In this respect, cocultures of hepatocytes with primitive biliary cells may be of importance. The suitability of this model has been shown by the elucidation of the hepatotoxic properties of the antiepileptic drug sodium VP ***(46)***. Indeed, in rats VP is metabolized by CYP2B1 to 4-ene VP and 2,4-diene VP ***(125)***, both known to be strong inducers of microvesicular steatosis and potent inhibitors of fatty acid β-oxidation. In cocultures, VP was found to be a potent inducer of CYP2B1, and the same results could also be reproduced in vivo ***(46)***. Thus, VP induces, both in vivo and in vitro, its own metabolism, leading to an overexposure of the liver cells to toxic unsaturated VP metabolites. Several other examples have been reported showing results in vitro similar to those in vivo ***(126,127)***.

## 4. Conclusion

Drug metabolism research is a key event in the discovery and development of new pharmaceuticals. For this purpose, hepatocyte-based in vitro models have proved very useful. In particular, hepatocyte suspensions and short-term cultures of hepatocytes have been applied with success in establishing the biotransformation pathways of new drugs in the liver, ranking compounds within a particular chemical class, elucidating the use of molecular mechanism of hepatotoxicity, and investigating species differences.

Phenotypic changes and rapid losses of xenobiotic-metabolizing enzymes of the hepatocytes may, in some cases, lead to erroneous results. Many potential ways to overcome this problem have been investigated, including different culture media, using additives, culturing on a substratum of components of the ECM or on microcarriers, or coculturing with other cell types ***(3,6,15,67,128)***. However, each of these methods has achieved only limited success. Therefore, new attempts are now focused on the use of specific molecules in the culture medium, aimed at regulating signaling pathways important in cell-cycle progression and apoptosis ***(16,17,29)***. Thus, development and use of long-term cultures of functionally stable hepatocytes remains a major goal.

The protocols used for the isolation of hepatocytes and their short-term culture differ considerably among laboratories and validated protocols do not yet exist ***(67,129)*** (*see* Chapter 26 for methods for the isolation of rat hepatocytes). At present, only the use of short-term hepatocyte cultures in unscheduled DNA synthesis assays,

to detect genotoxic substances, has been officially accepted ***(111)***, but it is clear that more can be accomplished. It seems quite feasible to expand the actual acute toxicity testing of compounds in vitro (by hepatocyte suspension and short-term cultures) to chronic toxicity testing in vitro, by improving the existing, more sophisticated long-term culture models debscribed in **Subheading 2.2.**

To avoid potential interspecies differences, human instead of animal in vitro models should be used to demonstrate the safety of a potential drug. As such, human hepatocytes and their cultures have proven most appropriate to predict drug metabolism and hepatotoxicity ***(2,3,10,67,130,131)*** (*see* Chapter 32 for methods for the preparation and culture of human hepatocytes). However, because the availability of human liver is limited, the use of human hepatocytes is very restricted and thus not suited for routine testing. In addition, the preparation of good hepatocytes from human tissue has often been problematic ***(132,133)***, but new techniques for the isolation of human hepatocytes from liver biopsies have been described ***(134)***. Furthermore, cryopreservation of hepatocytes is difficult and is always accompanied by a loss of cell viability, decreasing biotransformation capacity and in the ability to attach to culture dishes ***(10)***. Moreover, procedures for cryopreservation have not yet been standardized ***(135)***, although a consensus has been reached on certain aspects of cryopreservation ***(136)***. At present, cryopreserved human hepatocytes seem to be applicable only for short-term biotransformation and toxicity studies ***(137,138)***. Therefore, it is often more practical to select a species that has a pattern of metabolism similar to or close to that of humans. The use of hepatocyte suspensions and their cultures can thus help identify species suitable for further investigation in vivo as well as in vitro ***(10)***. However, it remains a challenge to develop better protocols for cryopreservation of human, dog, and monkey hepatocytes, the latter often being used in toxicology as the second nonrodent species.

Future strategies to overcome the shortage of human hepatocytes could be the use of human bone marrow stem cells. Indeed, multipotent adult progenitor cells isolated from the bone marrow are self-proliferative and have been shown to differentiate into hepatocyte-like cells ***(139,140)***. Therefore, they could provide an indefinite source of human hepatoyctes for toxicological studies. However, the isolation (*see* Chapter 30), cultivation, and characterization of these stem cell-derived hepatocytes are still in a fundamental phase and need to be further developed and studied in depth before such cells can be considered as alternatives to animal experimentation.

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# 26

## Isolation of Rat Hepatocytes

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**Summary**

In vitro models, based on liver cells or tissues, are indispensable in the early preclinical phase of drug development. An important breakthrough in establishing cell models has been the successful high-yield preparation of intact hepatocytes. In this chapter, the practical aspects of the two-step collagenase perfusion method, modified from the original procedure of Seglen, are outlined. Although applicable to the liver of various species, including human, the practical aspects of the method are explained here for rat liver. Critical parameters for the successful isolation of primary rat hepatocytes are highlighted and a troubleshooting guide is provided. In addition, a new development based on the inhibition of histone deacetylase activity is presented. This approach allows inhibition of cell-cycle reentry during hepatocyte isolation, a process known to underlie the dedifferentiation process of cultured hepatocytes.

**Key Words:** Hepatocyte; isolation; liver; two-step collagenase perfusion; cell cycle; histone deacetylase.

### 1. Introduction

The liver is the main organ involved in the metabolism and toxicity of xenobiotics. Hence, isolated hepatocytes from various species, including human, and their cultures constitute attractive in vitro models for pharmacotoxicological studies ***(1)*** (*see* Chapter 25).

Initial attempts to isolate adult parenchymal cells by mechanical methods were not very successful and were more concerned with cell yield than with cell integrity, viability, and functionality ***(2)***.

Major progress was made by the introduction of enzymes as dissociating agents, such as collagenase and hyaluronidase. In 1969, Berry and Friend ***(3)*** established an *in situ* collagenase perfusion technique for rat liver to obtain a high yield of viable hepatocytes. An important modification, introduced by Seglen ***(4,5)***, was the supplementation of the collagenase solution with $Ca^{2+}$ in a second perfusion step. This two-step

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collagenase procedure is still used today with only minor modifications. Usually, a perfusion with $Ca^{2+}$-free buffer is carried out first to attack the calcium-containing bridges between the cells, followed by a second perfusion with $Ca^{2+}$-containing buffer with collagenase. $Ca^{2+}$ is necessary to activate the collagenase, which digests the collagen matrix. This technique can be applied to the liver of various species, including human, and usually a cell population of hepatocytes with <5% nonparenchymal cells is obtained. Gebhardt et al. *(6)* have described a standard operational procedure for the isolation of rat hepatocytes.

## 2. Materials

1. Sodium pentobarbital (60 mg/mL) (stable for months at 4°C).
2. Sodium heparin (5000 IU/mL) (stable for months at 4°C).
3. Collagenase, type I (Clostridiopeptidase A) (stable for months if refrigerated dry). Warm to room temperature before opening the container.
4. Trypan blue: 0.4% (w/v) in 0.85% (w/v) NaCl.
5. 70% (v/v) Alcohol.
6. $Ca^{2+}$-free Krebs-Henseleit buffer (KHB), pH 7.4: 210 mL of 0.154 *M* $NaHCO_3$ saturated for 60 min with carbogen (95% $O_2$ and 5% $CO_2$), 1000 mL of 0.154 *M* NaCl, 40 mL of 0.154 *M* KCl, 10 mL of 0.154 *M* $KH_2PO_4$, and 10 mL of 0.154 *M* $MgSO_4$ all in dd$H_2O$. Sterilize the buffer by passing through a 0.22-µm filter; and it can be stored at 4°C for 6 mo.
7. $Ca^{2+}$-containing KHB, pH 7.4: 1270 mL of $Ca^{2+}$-free KHB and 30 mL of 0.110 *M* $CaCl_2$ in dd$H_2O$. Sterilize the buffer by passing through a 0.22-µm filter; it can be stored at 4°C for 6 mo.
8. HEPES buffer, pH 7.65: 0.80% (w/v) NaCl, 0.01% (w/v) $Na_2HPO_4 \cdot 12H_2O$, 0.02% (w/v) KCl, and 0.038% (w/v) HEPES in Millipore-quality water. Sterilize the buffer by passing through a 0.22-µm filter; it can be stored at 4°C for 6 mo.
9. Leibovitz medium: 1.47% (w/v) Leibovitz medium and 0.20% (w/v) bovine serum albumin (BSA) in Millipore-quality water. Sterilize the medium by passing through a 0.22-µm filter; it can be stored at 4°C for 6 mo.
10. Standard medium: Dulbecco's modified Eagle's medium, containing 4.5 mg/mL of glucose and 0.584 mg/mL of L-glutamine. This sterile medium can be stored for 6 mo at 4°C.
11. Wash medium: standard medium supplemented with 7.3 IU/mL of benzyl penicillin, 50 µg/mL of streptomycin sulfate, 50 µg/mL of kanamycin monosulfate, and 10 µg/mL of sodium ampicillin. This medium is sterilized and can be stored for 7 d at 4°C.
12. Phosphate-buffered saline, pH 7.65: 0.28% (w/v) NaCl, 0.02% (w/v) KCl, 0.31% (w/v) $Na_2HPO_4 \cdot 12H_2O$ and 0.02% (w/v) $KH_2PO_4$ in Millipore-quality water. Sterilize the buffer by passing through a 0.22-µm filter; it can be stored at 4°C for 9 mo.
13. Perlon filter (63 µm) and 0.22-µm filter.
14. Sterile volumetric pipets.
15. Glass cannula.
16. Bile cannula (i.d. of 0.28 mm, o.d. of 0.061 mm).
17. Hemocytometer.
18. Two 50-mL sterile centrifuge tubes.
19. Perfusion apparatus (**Fig. 1**).
20. Laminar flow cabinet.
21. Inverse-phase light microscope.
22. Perfusion pump.

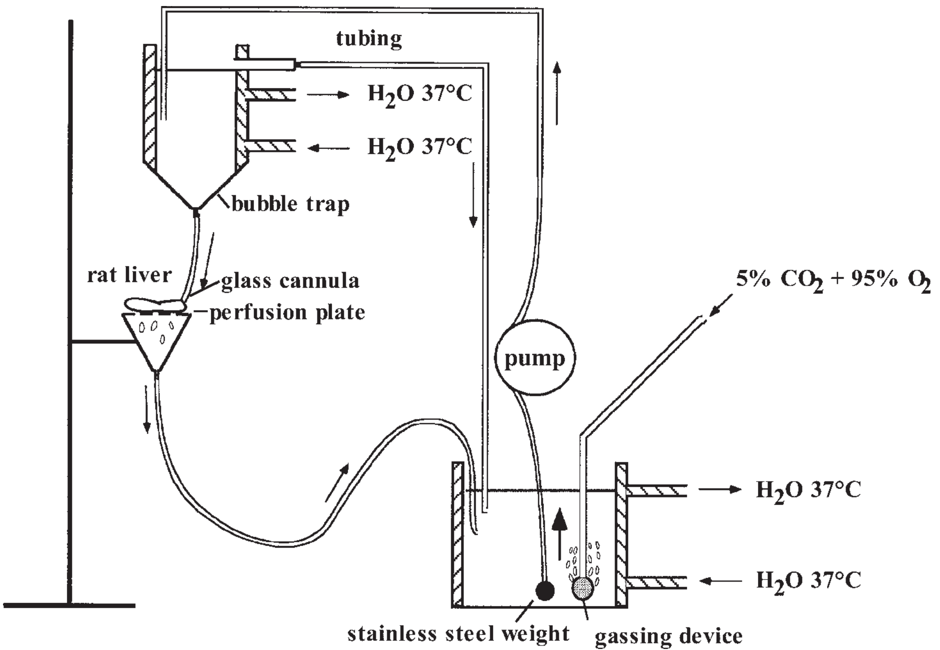


Fig. 1. Schematic representation of perfusion apparatus.

23. Centrifuge.
24. Thermostated bath.

## 3. Methods

### 3.1. Sterilization of Perfusion Apparatus

1. Sterilize the perfusion apparatus with 400 mL of denatured alcohol (70%), and circulate for 15 min.
2. Rinse the apparatus three times with sterile Millipore-quality water (10 min/circulation).

### 3.2. Isolation of Rat Liver (see Note 1)

1. Isolate hepatocytes from male Sprague-Dawley rats (±200 g) that have access to water and food ad libitum (*see* **Note 2A**).
2. Anesthetize a rat by injecting sodium pentobarbital solution (0.1 mL/100 g of body wt) intraperitoneally (*see* **Note 3**).
3. Shave the rat's abdomen, disinfect with alcohol, and immobilize the rat on a surgery table.
4. Open the abdomen by making a U-shaped incision.
5. Ligate the bile duct twice, close the lower ligature, insert the bile cannula, and fix it by closing the upper ligature.
6. Ligate the vena cava once and the vena porta twice.

7. Inject 1 mL of freshly prepared heparin solution (0.1 mL of heparin [5000 IU/mL]/ 2.4 mL of physiological saline) into the vena saphena medialis. The final concentration of heparin is 200 IU/mL.
8. Close the lower ligature of the vena porta, insert the glass cannula into the vena porta, and close the upper ligature.
9. Close the ligature of the vena cava.
10. Remove the liver from the rat and rinse it with $Ca^{2+}$-free KHB.

### *3.3. Two-Step Perfusion of the Rat Liver* (see *Notes 2, 4, and 5*)

1. Circulate 250 mL of $Ca^{2+}$-free KHB in the perfusion apparatus thermostated at 42°C (to obtain 37°C in the liver). Gas it with 5% $CO_2$ and 95% $O_2$.
2. Transfer the excised rat liver to a perfusion plate and connect the glass cannula, already inserted in the vena porta (perfusion rate: 40–50 mL/min; temperature: 37°C). The connecting tubes must be completely free of air bubbles.
3. For the first perfusion step, remove the blood from the liver with 100 mL of $Ca^{2+}$-free KHB, and circulate the remaining 150 mL of $Ca^{2+}$-free KHB for 15 min. If the liver does not blanch within a few seconds, it should be discarded.
4. For the second perfusion step, dissolve 18,400 digestion units of type I collagenase in 10 mL of gassed $Ca^{2+}$-containing KHB. Sterilize the solution by passing it through a 0.22-µm filter, and add it to the 150 mL of $Ca^{2+}$-free KHB in the perfusion apparatus. The final concentration of collagenase is 115 collagenase digestion units/mL. Circulate for 25 min.
5. Disconnect the perfused rat liver from the perfusion apparatus, and transfer it to a Petri dish filled with Leibovitz medium.

### *3.4. Purification of Rat Hepatocytes*

1. Open the Glisson's capsule and suspend the cells in Leibovitz medium.
2. Filter the resulting cell suspension through a sterile Perlon filter (63 µm).
3. Allow the cells to sediment for 15 min.
4. Remove the supernatant, wash the cells with 40 mL of HEPES buffer and dispense the cell suspension into two sterile 50-mL centrifuge tubes.
5. Centrifuge the cell suspension for 1 min at 63*g*.
6. Remove the supernatant and wash the cell pellet with 20 mL of HEPES buffer.
7. Centrifuge the cell suspension for 1 min at 63*g*.
8. Remove the supernatant and wash the cell pellet with wash medium (20 mL/tube).
9. Centrifuge the cell suspension for 1 min at 63*g*.
10. Remove the supernatant and resuspend the cell pellet in 100 mL of wash medium.

### *3.5. Determination of Cell Viability* (see *Note 6*)

1. Mix 600 µL of $Ca^{2+}$-containing KHB with 200 µL of Trypan blue solution and 400 µL of cell suspension.
2. Count the viable (white) and dead (blue) cells in a hemocytometer under a light microscope (magnification of 10 × 20). Usually, four fields are counted per chamber.
3. Calculate the mean number of viable and dead cells as follows:

$$\text{Viability (\%)} = (\text{no. of viable cells} \times 100)/[(\text{no. of viable cells}) + (\text{no. of dead cells})]$$

$$\text{Cell concentration (cells/mL)} = [(\text{no. of viable cells}) \times 3 \times 1000]/0.1$$

in which 3 = the dilution factor of the cell suspension, 1000 = the conversion factor from cubic millimeters to mililiters, and 0.1 = the volume contained by one field of the hemo-

cytometer. Only cell suspensions with a viability of 80% or more are used for in vitro purposes.

### *3.6. New Development: Prevention of G0/G1 Transition-Regulating Protein Expression During the Hepatocyte Isolation Procedure*

#### *3.6.1. Background*

The progressive loss of differentiated liver-specific functions and the limited life span of hepatocytes in culture ***(1,7)*** substantially limit their applicability. Initially, dedifferentiation was attributed to the use of inadequate culture techniques. Therefore, the first strategies pursued to overcome this problem were aimed at mimicking the in vivo hepatocyte microenvironment, by introducing cell–cell contacts (e.g., coculture with primitive biliary epithelial cells) and cell–matrix interactions (e.g., collagen-gel sandwich configuration) ***(1,7)***. However, evidence now exists to assume that the trigger for dedifferentiation initiates during the isolation of hepatocytes from the liver ***(8,9)***. During this procedure, cells spontaneously reenter the cell cycle, owing to altered gene expression patterns ***(10–12)***. This knowledge prompted us to investigate whether this transition from the G0 into the G1 phase of the cell cycle could be prevented by interfering with the histone acetylation/deacetylation process, which determines, to a large extent, the transcriptional activity of specific chromatin regions. For this purpose we used Trichostatin A 7-[4-(dimethylamino)phenyl]-*N*-hydroxy-4,6-dimethyl-7-oxo-2,4-heptadienamide (TSA) (**Fig. 2**), a prototype inhibitor of histone deacetylase activity with marked antiproliferative and prodifferentiating properties in a number of cancer cell lines in vitro ***(13–15)***.

#### *3.6.2. Practical Aspects*

TSA (purity ≥ 98%) was purchased from Sigma (St. Louis, MO) and dissolved in ethanol to a final concentration of 30 m*M*. Final ethanol concentrations in the media did not exceed 0.0033% (v/v) and did not affect any of the parameters investigated. All media used during the two-step perfusion of the liver (*see* **Subheadings 2.** and **3.3.**) were supplemented with 1 µ*M* TSA immediately before use. Perfusion of the rat liver was carried out as described in **Subheading 3.3.**

On perfusion of the liver in the constant presence of TSA (1 µ*M*), c-jun RNA and proteins, a major hallmark for G0/G1 transition, could not be detected. Moreover, once in culture, in the presence of TSA, these cells failed to respond to the mitogenic signals of epidermal growth factor, as evidenced by the absence of cyclin D1 protein, which normally is typically expressed after passage of the mid-to-late G1 restriction point ***(16)***.

Thus, TSA interferes with the G0/G1 transition of hepatocytes, and this process involves remodeling of the chromatin structure, because the nuclei of the hepatocytes were enriched in hyperacetylated histones H3 and H4 ***(16)***. Whether this early inhibition of c-jun induction contributes to the prevention of a dedifferentiation program that is initiated during hepatocyte isolation remains, at present, unclear. However, these findings might open new perspectives for further improvement of the currently applied hepatocyte isolation procedure and culture techniques.

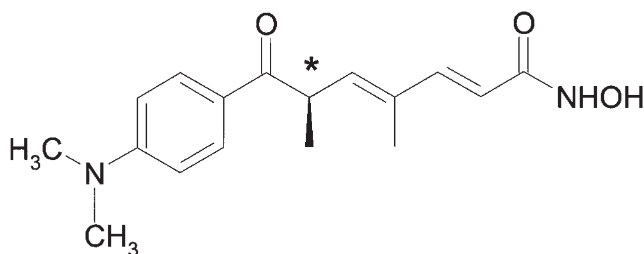


Fig. 2. Chemical structure of Trichostatin A (TSA). *Presence of a chiral center.

## 4. Notes

1. The method for the isolation of primary rat hepatocytes may be an important source of variation among results obtained from different laboratories using hepatocyte cultures. Although a validated standardized procedure does not yet exist, some recommendations for the isolation of rat hepatocytes have been presented in the report of the ECVAM Workshop 1 ***(17)***. Gebhardt et al. ***(6)*** have also published the standard operational procedure for the isolation of rat hepatocytes.
2. From our own experience, it appears that the following variables may be of importance:
   a. With respect to the cell source, it is recommended that the same strain, age, and gender of rats be used. Attention should also be paid to the health condition of the rats as well as to the food composition, feeding schedule, type of bedding, constant light/dark cycle, and environmental stress factors.
   b. With respect to the two-step perfusion technique, these factors should be considered:
      i. Before cannulation, heparin should be administered, in order to prevent coagulation of blood in the liver.
      ii. One must be aware whether $Ca^{2+}$-chelating agents are present in the first perfusate. If they are, their concentration and type should be known.
      iii. The $Ca^{2+}$ concentration in the second perfusate should be rigorously constant during all experiments.
      iv. Perfusion time should be kept as short as possible, and perfusion should be stopped as soon as the liver has softened sufficiently (constant time length or visual stop).
      v. The type, quality, activity, and storage conditions (dry at –20°C) of the collagenase used are important parameters. The enzyme activity and specificity of collagenase may show appreciable variations among different vendors or even among different batches from the same vendor. It is therefore recommended that several different batches of collagenase be tested in order to determine which one best preserves the function to be assessed in the isolated hepatocytes. Usually, collagenase activity is tested via the isolation of hepatocytes from rat liver.
      vi. The presence of other dissociating enzymes, such as hyaluronidase or trypsin, is discouraged.
      vii. The composition of perfusion buffers and their pH (7.4), level of oxygenation, temperature (37°C in liver), and osmolarity (300 mosM/L) must be kept constant during all experiments.

**Table 1**
**Troubleshooting of the Two-Step Collagenase Perfusion of Liver**

| Problem | Cause | Solution |
|---|---|---|
| Poor perfusion | Air bubbles in liver | Avoid bubbles during portal vein cannulation and transfer to the perfusion apparatus. A bubble trap is necessary. |
| | Particulates in liver | Filter the buffers and enzymes. |
| | Liver blood clots | Use heparin; flush the liver rapidly and completely to remove endogenous blood. |
| Incomplete digestion of liver | Poor perfusion | *See* poor perfusion. |
| | Insufficient enzyme concentration | Increase the amount or specific activity of collagenase. |
| | No $Ca^{2+}$ in buffer | Add $Ca^{2+}$ to 5 m*M*. |
| Poor recovery of cells | Large portions of nonperfused liver | *See* poor perfusion. |
| | Incomplete digestion | *See* incomplete digestion of liver. |
| | Low number of viable cells | Try different types or batches of collagenase; decrease the amount of collagenase; during perfusion, keep the liver moist. |
| | Cell clumping | Try adding of DNase I. |
| | Gross microbial contamination | Clean the perfusion apparatus; prepare fresh sterile buffers. |

viii. The use of either carbogen gassing (95% $O_2$ + 5% $CO_2$) in the case of $HCO_3$ buffers or HEPES to maintain the buffer pH at 7.4–7.5 is preferred. The pH can be finally adjusted with 0.1 *N* NaOH.
ix. The type of tubing should be compatible with the biological material and have a low potential for protein binding to its surface. The tubings and perfusion apparatus must be cleaned and sterilized before use.
x. The temperature, pH, and composition of the dispersing medium for the freshly isolated hepatocytes (e.g., presence, concentration, and type of fetal calf serum and BSA) must be kept constant during all experiments.
xi. Washing and purification procedures of the cell suspension (centrifugation time; *g* value; number of washings; and composition, pH, osmolarity, and volume of washing buffers) must be kept constant during all experiments.

3. A deep anesthesia of the animal is required. Mostly, sodium pentobarbital is used, but there is a great need to look for alternatives, because of the known effects of barbiturates on the liver. The use of ether is strongly discouraged owing to its effects on the respiratory system and dosing problems.
4. Some general problems may occur during liver perfusion, including poor perfusion of the liver, incomplete digestion of the liver, or poor recovery of the cells. Causes and solutions are presented in **Table 1** *(18)*.
5. Liver perfusion can be carried out with either a nonrecirculating or a recirculating apparatus. The former technique is not influenced by the release of metabolic products from the liver (which continuously changes the composition of the perfusate). However, this system is inconvenient and expensive because large amounts of digesting enzyme are required. Therefore, a recirculating system (**Fig. 1**) is most commonly applied.
6. It is highly recommended that the suspensions of freshly isolated rat hepatocytes be used only if a minimum viability of at least 80% (as measured by exclusion of Trypan blue) and a minimum attachment yield of 80% are obtained immediately following isolation *(17)*.

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# 27

## Rat Hepatocyte Cultures

*Conventional Monolayer and Cocultures With Rat Liver Epithelial Cells*

**Tom Henkens, Tamara Vanhaecke, Peggy Papeleu, Greetje Elaut, Mathieu Vinken, Sarah Snykers, and Vera Rogiers**

**Summary**

Primary cultures of hepatocytes are useful tools for both short- and long-term pharmacotoxicological research. Under conventional conditions, isolated hepatocytes form a monolayer and survive for about 1 wk but lose some liver-specific functions, including xenobiotic biotransformation. In comparison with the conventional monolayer culture model, cocultures with rat liver epithelial cells (RLECs) have an extended life-span and better maintain their drug-metabolizing capacity, owing to the presence of cell–cell interactions. In this chapter, techniques for setting up conventional monolayer cultures and cocultures of hepatocytes with RLECs (including isolation, culture, and cryopreservation of RLECs) are described in detail. In addition, comments derived from our own experience are given for successfully culturing primary hepatocytes.

**Key Words:** Rat liver epithelial cells; primary hepatocytes; monolayer; coculture; differentiation; xenobiotic biotransformation.

### 1. Introduction

To be useful for both short- and long-term pharmacotoxicological research, hepatocytes must survive more than a few hours and maintain hepatic functions throughout culture time. Therefore, they need to attach to an appropriate support.

Under conventional conditions, hepatocytes form a monolayer and survive for about 1 wk. Following isolation (*see* Chapter 26), however, hepatocytes undergo phenotypical alterations, accompanied by a significant reduction or loss of some liver-specific functions, including important xenobiotic metabolic pathways (*see* Chapter 25). Despite these shortcomings, conventional monolayer cultures provide a good tool for drug metabolism studies and direct hepatotoxicity tests ***(1,2)***.

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Because cell–cell interactions are considered to contribute substantially to the maintenance of a differentiated phenotype of cultured hepatocytes, cocultures have been established with other cell types, such as sinusoidal endothelial cells *(3)*; epithelial cell lines *(4)*; mesenchymal cell lines *(5)*; fibroblastic cell lines *(6,7)*; rat liver epithelial cells (RLECs) *(8)*; and, more recently, bone marrow-derived stromal cells *(9)*. When cocultured, hepatocytes survive longer and maintain better their drug-metabolizing capacity, because this model mimics better the in vivo situation, compared with the conventional monolayer culture model. Despite the fact that cocultures are more sophisticated and labor intensive, they represent a very good model for long-term toxicity and biotransformation testing *(1,10)*.

In this chapter, techniques for conventional monolayer culture and coculture of hepatocytes with RLECs are described in detail. They are based on the work of Guguen-Guillouzo *(11)*, Guillouzo et al. *(12)*, and Williams et al. *(13)*.

## 2. Materials

### 2.1. Conventional Monolayer Cultures

1. Standard medium, pH 5.2: 75% Minimum Essential Medium, 25% Medium 199, bovine serum albumin (fraction V, minimum of 96%) (1 mg/mL), and bovine insulin (from bovine pancreas prepared from starting material with an activity of 27.8 IU/mg) (10 µg/mL). Sterilize this medium by passing through a 0.22-µm filter; it can be stored for 3 mo at –20°C.
2. T0 medium: standard medium supplemented with 10% (v/v) fetal bovine serum (FBS), $NaHCO_3$ (2.2 mg/mL), benzyl penicillin (7.3 IU/mL), streptomycin sulfate (50 µg/mL), kanamycin monosulfate (50 µg/mL), and sodium ampicillin (10 µg/mL). Four hours after cell seeding, the medium is renewed with T0 medium (37°C) supplemented with $7 \times 10^{-5}$ *M* hydrocortisone hemisuccinate, whereas 24 h after seeding and every day thereafter, serum-free T0 medium (37°C) supplemented with $7 \times 10^{-5}$ *M* hydrocortisone hemisuccinate is used. Prepare all media sterilely; they can be stored for 7 d at 4°C.
3. Thioglycollate medium: yeast extract (5 g/L), tryptone (15 g/L), glucose (5.5 g/L), sodium thioglycollate (0.5 g/L), NaCl (2.5 g/L), L-cystine (0.5 g/L), resazurine (0.001 g/L), and agar (0.5 g/L).
4. Sterile plastic Petri dishes (15, 10, or 6 cm in diameter).
5. Sterile volumetric pipets.
6. Sterile Pasteur pipets.
7. Laminar-airflow cabinet.
8. Incubator (37°C, water jacketed, humidified atmosphere of 95% air and 5% $CO_2$).
9. Thermostated bath (37°C).
10. Phase-contrast inverse light microscope.

### 2.2. Cocultures With RLECs

#### 2.2.1. Isolation of RLECs

1. Two sterile 100-mL bottles.
2. Two sterile nylon filters (80 µm) in funnels.
3. Sterile glass Petri dish (10 cm in diameter).
4. Sterile magnet.
5. Sterile plastic 50-mL centrifuge tubes.
6. Sterile plastic Petri dishes (4 cm in diameter).

7. Fine platinum needle.
8. Sterile volumetric pipets.
9. Sterile Pasteur pipets.
10. Laminar-airflow cabinet.
11. Incubator (37°C, water jacketed, humidified atmosphere of 95% air and 5% $CO_2$).
12. Thermostated bath (37°C).
13. Centrifuge.
14. Phase-contrast inverse light microscope.
15. Sterile phosphate-buffered saline (PBS), pH 7.65 (1 L): 0.28 % (w/v) NaCl, 0.31% (w/v) $Na_2HPO_4 \cdot 12H_2O$, 0.02% (w/v) KCl, and 0.02% (w/v) $KH_2PO_4$ in Millipore-quality water; sterilize by passing through a 0.22-µm filter.
16. Trypsin solution: 0.25% (w/v) trypsin (from porcine pancreas, 12,700 U/mg of protein) in PBS; sterilize by passing through a 0.22-µm filter (approx 67 mL/g of liver).
17. Williams' E medium; it can be stored for 6 mo at 4°C.
18. Williams' E medium (200 mL) for RLECs supplemented with antibiotics (as described for T0-medium in **Subheading 2.1., item 2**) and L-glutamine (0.1 mg/mL) (WM). Prepare this medium sterilely; it can be stored for 7 d at 4°C.
19. WM (500 mL) supplemented with 10% (v/v) FBS (WMF). Prepare this medium sterilely; it can be stored for 7 d at 4°C.

### 2.2.2. Culture of RLECs

1. Sterile flasks (75 $cm^2$) treated for tissue culture.
2. Sterile volumetric pipets.
3. Sterile Pasteur pipets.
4. Incubator (37°C, water jacketed, humidified atmosphere of 95% air and 5% $CO_2$).
5. Thermostated bath (37°C).
6. Laminar-airflow cabinet.
7. Phase-contrast inverse light microscope.
8. Sterile PBS (*see* **Subheading 2.2.1., item 15**).
9. Sterile trypsin-EDTA solution: 0.05% (w/v) trypsin, 0.53 m*M* sodium EDTA in Hank's Balanced Salt Solution (HBSS) without $CaCl_2$, $MgCl_2$ and $MgSO_4$.
10. WMF (*see* **Subheading 2.2.1., item 19**).

### 2.2.3. Cryopreservation of RLECs

1. Liquid nitrogen.
2. Sterile 2-mL Nunc-tubes.
3. Sterile plastic 15-mL centrifuge tubes.
4. Sterile volumetric pipets.
5. Sterile Pasteur pipets.
6. Laminar-airflow cabinet.
7. Incubator (37°C, water jacketed, humidified atmosphere of 95% air and 5% $CO_2$).
8. Thermostated bath (37°C).
9. Centrifuge.
10. Phase-contrast inverse light microscope.
11. Sterile PBS (*see* **Subheading 2.2.1., item 15**).
12. Sterile trypsin-EDTA (*see* **Subheading 2.2.2., item 9**).
13. WMF (*see* **Subheading 2.2.1., item 19**).
14. WMF supplemented with 20% (v/v) dimethyl sulfoxide (DMSO).

#### 2.2.4. Coculture

1. The same materials as for the conventional monolayer cultures (*see* **Subheading 2.1.**).
2. Sufficient confluent layers of RLECs (from not later than the 30th passage) for the coculture experiment planned.

## 3. Methods

### 3.1. Conventional Monolayer Cultures (see Notes 1–4)

1. Prepare the media, check their sterility by adding 1 mL of the medium to 25 mL of autoclaved thioglycollate medium and incubating this mixture at 37°C. After 2 d, investigate thioglycollate media for contamination. Discard if cloudy.
2. Before use, warm the media for about 30 min in a thermostated bath (37°C).
3. Isolate rat hepatocytes as described in Chapter 26.
4. Seed the hepatocytes at a density of $1.6 \times 10^6$ cells/28.3-cm$^2$ Petri dish, $4.4 \times 10^6$ cells/78.5-cm$^2$ Petri dish or $10 \times 10^6$ cells/177-cm$^2$ Petri dish in 4, 11, and 25 mL T0 medium, respectively.
5. Allow cell attachment by incubating the hepatocyte cultures at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$.
6. Four hours after cell seeding, renew the medium with 4 mL (28.3-cm$^2$ Petri dishes), 11 mL (78.5-cm$^2$ Petri dishes) or 25 mL (177-cm$^2$ Petri dishes) of T0-medium (37°C) supplemented with $7 \times 10^{-5}$ *M* hydrocortisone hemisuccinate.
7. Renew the medium every day thereafter with the same amounts of serum-free T0 medium (37°C) supplemented with $7 \times 10^{-5}$ *M* hydrocortisone hemisuccinate (*see* **Note 3**).

### 3.2. Cocultures With RLECs

#### 3.2.1. Isolation of RLECs

1. Weigh a sterile 100-mL bottle with lid containing 50 mL of sterile PBS.
2. Decapitate ten 8- to 10-d-old Sprague-Dawley rats.
3. Open the abdominal walls of the animals and remove the livers.
4. Collect the livers in the 50 mL of PBS.
5. Reweigh the bottle; the difference between this weight and that determined in **step 1** is the mass of collected liver.
6. Remove the PBS and transfer the livers to a sterile 10-cm-diameter glass Petri dish.
7. Add 8 mL of sterile PBS and cut the livers into small pieces.
8. Transfer these to the 0.25% (w/v) trypsin solution (20 mL/g of collected liver) in a 100-mL sterile bottle, add a sterile magnet, seal the bottle, and stir for 10 min on a magnetic stirrer.
9. Leave for a few minutes.
10. Remove the trypsin solution carefully with a 10-mL sterile pipet.
11. Add 20 mL of trypsin solution/g of collected liver and stir for 15 min.
12. Leave for a few minutes and filter the supernatant through an 80-µm nylon filter into a 50-mL plastic centrifuge tube (save the material that does not pass through the filter).
13. Centrifuge the filtrate for 5 min at 626*g*.
14. Remove the supernatant, leaving approx 10 mL above the pellet.
15. Dilute the pellet to 35 mL with WM and resuspend.
16. Centrifuge for 5 min at 626*g*.
17. Remove the supernatant completely and resuspend the pellet in 1.66 mL of WM/g of collected liver (=A).

18. To the liver material that did not pass through the filter (**step 12**), add 60 mL of trypsin solution and stir for 15 min.
19. Repeat **steps 12–17** (=B).
20. Combine A and B. If an aggregate forms, refilter.
21. Prepare cultures of four different dilutions. For example, for 3 g of collected liver in a total volume of 10 mL of WM (A + B), place 0.25, 0.50, 0.75, and 1.00 mL of A + B mixture into four different 4-cm-diameter Petri dishes. Dilute each to a total volume of 4.00 mL with WMF.
22. Incubate the cultures for 20 min (=first series).
23. Transfer the medium of the first series of cultures into new Petri dishes, make up to 4 mL with WMF, and incubate for 20 min (=second series).
24. Renew the medium of the first series with 4 mL of WMF and incubate for 24 h.
25. After 20 min, transfer the medium of the second series into new Petri dishes, dilute to 4 mL with WMF, and incubate for 20 min (=third series).
26. Renew the medium of the second series with 4 mL of WMF and incubate for 24 h.
27. After 2 h, transfer the medium of the third series into new Petri dishes, dilute to 4 mL with WMF, and incubate for 24 h (=fourth series).
28. Renew the medium of the third series with 4 mL of WMF and place in an incubator for 24 h.
29. After 24 h, renew the medium of all Petri dishes with WMF.
30. Renew the medium thereafter every 48 h until colonies of RLECs are visible under a microscope (after a few weeks).
31. Scan the Petri dishes every day for fibroblasts. When fibroblasts are present, scrape these off using a heated platinum needle.
32. When the colonies are large enough (i.e., >100 cells/colony), transfer the RLECs into new 4-cm-diameter Petri dishes and let these form new colonies by repeating **steps 29** and **30**.
33. After 2 to 3 mo, a pure population of good dividing RLECs is obtained. Subculture further in 75-cm$^2$ culture flasks.

#### *3.2.2. Culture of RLECs*

1. Add to a 75-cm$^2$ culture flask 10 mL of WMF at 37°C.
2. Transfer the RLECs from one Petri dish (as obtained in **Subheading 3.2.1., step 33**) into a culture flask (=replication number 1).
3. Incubate the cells for 24 h.
4. Renew with 10 mL of WMF at 37°C.
5. Renew the medium thereafter every 2 d with 10 mL of WMF until a confluent layer of RLECs is obtained (approx 8 d).
6. Remove the medium.
7. Add 5 mL of sterile PBS (37°C) per flask, shake gently, and remove the PBS.
8. Add 5 mL of sterile trypsin-EDTA (37°C) per flask, shake gently, and leave the flask sealed for 5 min at room temperature.
9. Remove the trypsin-EDTA, reseal the flask, and place it for approx 15 min in the incubator.
10. Detach the RLECs by tapping the flask. Confirm that the cells have detached by examining under a microscope (i.e., they are in floating suspension).
11. Add 20 mL of WMF at 37°C per flask and transfer 10 mL of cell suspension into two new sterile culture flasks (=replication number 2). Mix gently to spread the cell suspension over the flask.
12. Repeat **steps 3–11** until enough confluent layers of RLECs are obtained to set up the coculture experiment planned or cryopreserve the RLECs in Nunc tubes.

### 3.2.3. Cryopreservation of RLECs (see **Note 5**)

1. Remove the medium from a confluent layer of RLEC.
2. Wash the cells with 5 mL of sterile PBS (37°C) per flask.
3. Add 5 mL of sterile trypsin-EDTA (37°C) per flask, shake gently, and leave the flask closed for 5 min at room temperature.
4. Remove the trypsin-EDTA, seal the flask, and place it in an incubator for approx 15 min.
5. Detach the RLECs by tapping the flask. Check under the microscope that the cells have detached (i.e., they are floating in suspension).
6. Add 10 mL of WMF per flask.
7. Transfer the cell suspension into sterile plastic 15-mL centrifuge tubes.
8. Centrifuge for 5 min at 626*g*.
9. Resuspend the pellet in 4 mL of WMF.
10. Homogenize well (by pipetting up and down), and divide the suspension among four sterile 2-mL Nunc tubes (1 mL of suspension/tube).
11. Add to each tube 1 mL of WMF supplemented with 20% (v/v) DMSO.
12. Seal the tubes and cryopreserve in liquid nitrogen (write the replication number on the tube).

### 3.2.4. Coculture (see **Notes 4** and **5**)

1. Follow **steps 1–5** in **Subheading 3.1.** for plating the hepatocytes.
2. After attachment of the hepatocytes (approx 3.5 h) do the following:
   a. Detach the confluent layers of RLECs from the bottom of the flasks as described in **Subheading 3.2.3., steps 1–5**.
   b. Prepare the cocultures as follows: add one-fourth, one-half, or the total amount of the cells derived from a 75-$cm^2$ flask of confluent RLECs suspended in 4, 11, or 25 mL of renewing medium to 28.3-, 78.5-, or 177-$cm^2$ Petri dishes, respectively. This consists of T0 medium (37°C) supplemented with $7 \times 10^{-6}$ *M* hydrocortisone hemisuccinate.
3. Every day thereafter, renew the medium with either 4, 11, or 25 mL of serum-free T0 medium (37°C) supplemented with $7 \times 10^{-6}$ *M* hydrocortisone hemisuccinate (*see* **Note 3**).

## 4. Notes

1. Owing to numerous factors, in part described in Chapter 25, high variability in the degree of differentiation and, consequently, in drug-metabolizing capacity is observed. To reduce variability, an ECVAM Task Force on Good Cell Culture Practice was initiated to define minimum requirements for quality standards to ensure reproducibility and reliability ***(14,15)***.
2. The composition of the medium as described in **Subheading 2.1., items 1** and **2** is suitable for general purposes. This medium when supplemented with epidermal growth factor has been successfully used to study hepatocyte proliferation ***(16)***. According to individual needs and the desired degree of differentiation of the hepatocytes, the composition of the media can be altered. Other media such as William's E and Modified Chee's medium also have been shown to be appropriate for cultivation of differentiated hepatocytes ***(17,18)*** (*see* also **Note 4**).
3. To prevent fungous infections of both monolayer cultures and cocultures, amphotericin B (0.25 μg/mL) can be added to the medium.
4. From the literature ***(10,12,19,20)*** and our own experience ***(17,21–24)*** additional important factors can be summarized as follows:

a. The composition of the culture media, in particular the serum concentration and the inclusion of hormones, growth factors, differentiation-inducing agents such as DMSO, inducers, and other soluble factors.
b. The pH and osmolarity of the culture media.
c. The type of plastic culture dishes or flasks used, their coating, and whether or not they underwent a special treatment for cell culture.
d. The cell density and extent of cell–cell interactions.
e. The oxygen supply during culture.
f. The presence of phenol red in the culture media and its interference with some assays.
g. The duration of exposure to substrates.
h. The presence of organic solvents in which water-insoluble substances are added and their effect on the end points being measured.
i. The use of appropriate control cultures.
j. The lack of standardized protocols used to study drug metabolism in cultured cells at the functional enzyme activity, protein, and mRNA levels.

5. Problems specific for cocultures of rat hepatocytes with RLECs are as follows:
   a. Poor characterization of the helper cells.
   b. Poor standardization of the isolation method of RLECs.
   c. Use of RLECs with a different replication number in different experiments.
   d. Contribution of the RLECs to the DNA and protein contents, which are used to normalize most biochemical and metabolic parameters.

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# 28

## Rat Hepatocyte Cultures

### *Collagen Gel Sandwich and Immobilization Cultures*

**Mathieu Vinken, Greetje Elaut, Tom Henkens, Peggy Papeleu, Sarah Snykers, Tamara Vanhaecke, and Vera Rogiers**

**Summary**

Mimicking the in vivo microenvironment is one of the current strategies to maintain liver-specific functionality in primary cultured hepatocytes for long periods. Freshly isolated hepatocytes entrapped in collagen gel type I (collagen gel immobilization culture) or sandwiched between two layers of hydrated collagen type I (collagen gel sandwich culture) are known to display liver-specific functions (e.g., biotransformation capacity) for more than 6 wk. We describe how to set up both types of organotypical hepatocyte culture systems. Besides a detailed protocol, we give some practical tips, taken from our own experience with long-term hepatocyte culture.

**Key Words**: Long-term culture; primary hepatocyte culture; collagen gel sandwich culture; collagen gel immobilization culture; cell–matrix interaction.

## 1. Introduction

It has become well established that the hepatic extracellular matrix (ECM) plays a major role in the modulation of several aspects of cellular function, such as morphogenesis and differentiation. Both integrin and nonintegrin receptors on the hepatocyte membrane interact with ECM compounds, including laminin, fibronectin, and collagen. It is thought that this ligand-receptor interaction activates intracellular signaling pathways and, consequently, liver-specific gene expression ***(1,2)***.

Based on this knowledge, several ECM-derived hepatocyte cultures mimicking the in vivo microenvironment have been developed. Both natural and synthetic polymers are used as scaffolds for hepatocytes. The latter includes matrices made of compounds such as poly(lactic-*co*-glycolic) acid ***(3)***, polyvinyl formal resin ***(4)***, and polyurethane foam ***(5)***. With respect to the drug-metabolizing capacity of hepatocytes cultured in these matrices, however, almost no data are available thus far ***(5–7)***. By contrast, hepa-

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tocytes cultured on natural ECM components have been studied in detail *(8–11)*. In particular, hepatocytes entrapped in a collagen gel type I or sandwiched between two layers of hydrated collagen type I are believed to mimic the in vivo situation. Within these cultures, hepatocyte polarity is restored, and liver-enriched transcription factors, including: CCAAT enhancer binding protein (CCEBP), D-binding protein (DBP) and hepatocyte nuclear factors (HNFs), are expressed at a high level *(10,11)*. The latter correlates with the maintenance of liver-specific functions such as the secretion of albumin, transferrin, fibrinogen, urea, and bile salts, for more than 6 wk *(12–15)*. As for biotransformation capacity, it has been shown that both phase I cytochrome P450 and phase II glutathione-*S*-transferase biotransformation enzymes are generally better maintained in collagen gel cultures compared with the conventional monolayer culture *(8–13)*. Moreover, collagen gel cultures are suitable in vitro models for evaluating xenobiotics as potential inducers of biotransformation enzymes *(16,17)*.

In this chapter, the collagen gel sandwich culture model and its easy-to-apply modification, the immobilization culture, are described in detail (*see* **Subheadings 2.1.** and **3.1.**). The former technique is based on the work of Dunn et al. *(14)*, and the latter on that of Koebe et al. *(18)*. The collagen isolation method is a modification of the method of Elsdale and Bard *(19)*.

## 2. Materials

### 2.1. Preparation of Rat Tail Collagen Type I

1. 1% (w/v) NaCl in Millipore-quality water.
2. 3% (v/v) $CH_3COOH$ in Millipore-quality water.
3. 30% (w/v) NaCl in Millipore-quality water.
4. 5% (w/v) NaCl/0.6% (v/v) $CH_3COOH$ in Millipore-quality water.
5. 0.6% (v/v) $CH_3COOH$ in Millipore-quality water.
6. 1 m*M* HCl in Millipore-quality water.
7. Chloroform.
8. Liquid antiseptic soap: 4% (w/v) chlorhexidin digluconate.
9. Two forceps.
10. Four Petri dishes (15-cm diameter).
11. Four sterile compresses (5 × 5 cm).
12. Beaker (500 mL) with seal.
13. Sterile glass bottle (500 mL).
14. Six centrifuge bottles (250 mL).
15. Dialysis membranes (molecular weight cut off of 12–14,000; 29-mm diameter).
16. Cooled centrifuge.

### 2.2. Collagen Gel Sandwich Culture (see Notes 1–6)

1. Standard medium: Dulbecco's modified Eagle medium (DMEM) with glucose (4.5 mg/mL). The sterile medium can be stored for 6 mo at 4°C.
2. T0 medium: standard medium supplemented with 10% (v/v) fetal bovine serum (FBS), sodium benzyl penicillin (7.3 IU/mL), streptomycin sulfate (50 μg/mL), kanamycin monosulfate (50 μg/mL), sodium ampicillin (10 μg/mL), L-glutamine (292 mg/mL), insulin (from bovine pancreas, cell culture tested) (0.5 IU/mL), and glucagon (from porcine pancreas, cell culture tested) (0.007 μg/mL). Prepare this medium sterilely; it can be stored for 7 d at 4°C.

3. T4 medium: T0 medium supplemented with hydrocortisone sodium hemisuccinate (25 µg/mL), and amphotericin B (0.25 µg/mL). Prepare this medium sterilely; it can be stored for 7 d at 4°C.
4. D medium: T4 medium without FBS. Prepare this medium sterilely; it can be stored for 7 d at 4°C.
5. Wash medium: standard medium supplemented with antibiotics and L-glutamine as described for the T0 medium (*see* **item 2**). Prepare this medium sterilely; it can be stored for 7 d at 4°C.
6. Thioglycollate medium: yeast extract (5 g/L), tryptone (15 g/L), glucose (5.5 g/L), sodium thioglycollate (0.5 g/L), NaCl (2.5 g/L), L-cystine (0.5 g/L), resazurine (0.001 g/L), and agar (0.5 g/L).
7. Rat tail collagen gel type I (1.1 mg/mL): prepare the required concentration from the collagen gel type I stock solution by diluting with sterile 1 m*M* HCl solution. Prepare ex tempore.
8. 10X concentrated DMEM with 3.7% (w/v) $NaHCO_3$; sterilize this medium by passing through a 0.22-µm filter; it can be stored for 3 mo at 4°C.
9. Sterile plastic Petri dishes (35-mm and 6-, 10-, and 15-cm diameter).
10. Sterile centrifuge tubes (15 mL).
11. Sterile Pasteur pipets and volumetric pipets.
12. Laminar airflow cabinet.
13. Incubator (water jacketed, 37°C, humidified atmosphere of air containing 5% $CO_2$).
14. Thermostated bath (37°C).

### 2.3. Collagen Gel Immobilization Culture (see Notes 1–6)

1. Standard medium: DMEM with glucose (4.5 mg/mL). The sterile medium can be stored for 6 mo at 4°C.
2. T0 medium: standard medium supplemented with 5% (v/v) FBS, antibiotics, L-glutamine, insulin and glucagon, as described for the T0 medium under **Subheading 2.2., item 2**. Prepare this medium sterilely; it can be stored for 7 d at 4°C.
3. T30' medium: standard medium supplemented with 2.5% (v/v) FBS, antibiotics, L-glutamine, insulin and glucagon, as described for the T0 medium under **Subheading 2.2., item 2**. Prepare this medium sterilely; it can be stored for 7 d at 4°C.
4. T4 medium (*see* **Subheading 2.2., item 3**).
5. D medium (*see* **Subheading 2.2., item 4**).
6. Thioglycollate medium (*see* **Subheading 2.2., item 6**).
7. Rat tail collagen gel type I (1.63 mg/mL): prepare the required concentration as described under **Subheading 2.2., item 7**.
8. 10X concentrated DMEM with 3.7% (w/v) $NaHCO_3$; sterilize this medium by passing through a 0.22-µm filter; it can be stored for 3 mo at 4°C.
9. Equipment as described under **Subheading 2.2., items 9–14**, plus a cryostat and a cooling plate (15°C).

## 3. Methods

### 3.1. Preparation of Rat Tail Collagen Type I (see Note 7)

#### 3.1.1. Collection of Collagen Fibers

1. Collect the tails from male Sprague-Dawley rats (e.g., after isolation of the liver) and store them at –20°C.

2. Scrub the rat tails with liquid antiseptic soap.
3. Dissect the collagen fibers from a number of rat tails.
4. Collect these in 1% (w/v) NaCl, and wash them once in 1% (w/v) NaCl and twice in double distilled water.
5. Stir the collagen fibers in 3% (v/v) $CH_3COOH$ (200 mL/tail) overnight at 4°C.

#### 3.1.2. Purification

1. Carefully filter the collagen through four layers of sterile compresses.
2. Centrifuge the collagen at 11,000*g* for 2 h at 4°C.
3. Measure the volume of the supernatant, and add dropwise to the supernatant while stirring at 4°C 0.2 vol of 30% (w/v) NaCl.
4. Leave the collagen solution without stirring for at least 1 h at 4°C (or overnight).
5. Centrifuge the collagen at 1750*g* for 30 min at 4°C.
6. Wash the pellet twice with 5% (w/v) NaCl/0.6% (v/v) $CH_3COOH$ (200 mL/centrifuge bottle). Centrifuge after each wash for 30 min at 1750*g* at 4°C.
7. Dissolve the pellet in 0.6% (v/v) $CH_3COOH$ (50 mL/tail).
8. Stir the collagen solution overnight at 4°C.

#### 3.1.3. Dialysis

1. Transfer the collagen solution into dialysis membranes.
2. Dialyze against 10 vol of 1 m*M* HCl at 4°C for 20 h, and change the buffer every 4 h.
3. Centrifuge the collagen solution at 11,000*g* for 2 h at 4°C.

#### 3.1.4. Sterilization

1. Measure the volume of the supernatant and add 0.003 vol of chloroform.
2. Transfer the collagen solution into a 500-mL beaker with a seal.
3. Stir for 48 h at 4°C.

#### 3.1.5. Lyophilization

1. Transfer the collagen solution into a sterile 500-mL bottle and store at 4°C.
2. Lyophilize two 5-mL samples of the resulting collagen solution in order to determine the concentration.

### 3.2. Collagen Gel Sandwich Culture (see *Notes 8–10*)

1. Isolate rat hepatocytes as described in Chapter 26.
2. Precoat the culture dishes as follows:
    a. Place rat tail collagen gel type I (1.1 mg/mL), 10X concentrated DMEM, and a 15-mL sterile centrifuge tube on ice in a laminar airflow cabinet.
    b. In the 15-mL sterile centrifuge tube on ice, mix 1 part of the 10X concentrated DMEM with 10 parts of the collagen gel (1.1 mg/mL).
    c. Disperse 350 μL and 1.00, 2.75, and 6.25 mL of this mixture over 35-mm and 6-, 10-, and 15-cm diameter plastic Petri dishes, respectively. The mixture must evenly cover the complete surface of the Petri dishes. Avoid air bubbles.
    d. Transfer the precoated Petri dishes to an incubator. Precoated Petri dishes are stored for a minimum of 45 min and a maximum of 5 d in the incubator.
3. Seed the rat hepatocytes as follows:
    a. Warm the T0 and T4 media for about 30 min in a thermostated bath (37°C) before use.

b. Seed the rat hepatocytes at a density of $4 \times 10^5$ cells/mL of T0 medium in 2, 4, 11, and 25 mL in 35-mm and 6-, 10-, and 15-cm-diameter Petri dishes, respectively.
c. Four hours after seeding, renew the medium with 2, 4, 11, and 25 mL of T4 medium in the 35-mm and 6-, 10-, and 15-cm-diameter Petri dishes, respectively.

4. Twenty-four hours after cell seeding, add the second layer of rat tail collagen gel type I and complete the sandwich configuration as follows:
   a. Warm the wash medium and D medium for about 30 min in a thermostated bath (37°C) before use.
   b. Place rat tail collagen gel type I (1.1 mg/mL), 10X concentrated DMEM, and a 15-mL sterile centrifuge tube on ice in the laminar airflow cabinet.
   c. Wash the cells present in the 35-mm and 6-, 10-, and 15-cm-diameter Petri dishes twice with 1, 2, 5, and 10 mL of wash medium, respectively.
   d. In the 15-mL sterile centrifuge tube on ice, mix 1 part of the 10X concentrated DMEM with 10 parts of the collagen gel type I (1.1 mg/mL).
   e. Disperse 350 μL and 1.00, 2.75, and 6.25 mL of this mixture over 35-mm and 6-, 10-, and 15-cm-diameter plastic Petri dishes, respectively. The mixture must be evenly dispersed over the surface of the Petri dishes. Avoid air bubbles.
   f. Place the completed collagen gel sandwich cultures in the incubator for about 45 min.
   g. Add 2, 4, 11, and 25 mL of D medium to each of the 35-mm and 6-, 10-, and 15-cm-diameter Petri dishes, respectively.
5. Renew the medium every day thereafter with the same volume of D medium.

### *3.3. Collagen Gel Immobilization Culture* (see *Notes 8–10*)

1. Isolate rat hepatocytes as described in Chapter 26.
2. Cool the Petri dishes required on a cooling plate at 15°C.
3. Place a cell suspension of $1.6 \times 10^6$ cells/mL, rat tail collagen gel type I (1.63 mg/mL), 10X concentrated DMEM, and a 15-mL sterile centrifuge tube on ice in a laminar airflow cabinet.
4. In the 15-mL sterile centrifuge tube on ice, mix 1 part of the 10X concentrated DMEM with 10 parts of the collagen gel type I (1.63 mg/mL).
5. Mix on ice 350 μL and 1.00, 2.75, and 6.25 mL of this mixture with 350 μL, 1.00, 2.75, and 6.25 mL of the cell suspension, respectively, and disperse into 35-mm and 6-, 10-, and 15-cm-diameter cooled plastic Petri dishes, respectively.
6. Leave the Petri dishes on the cooling plate (15°C) for 15 min.
7. Place the collagen gel immobilization cultures in an incubator for 30 min.
8. Add 2, 4, 11, and 25 mL of T30' medium to 35-mm and 6-, 10-, and 15-cm-diameter Petri dishes containing the collagen gel immobilization cultures, respectively.
9. Four hours after seeding renew the medium with 2, 4, 11, and 25 mL of the T4 medium in the 35-mm and 6-, 10-, and 15-cm-diameter Petri dishes, respectively.
10. Renew the medium every day thereafter with the same volume of D medium.

## 4. Notes

1. Although the presence of the ECM is responsible for the long-term expression of liver-specific genes, the addition of soluble factors, in particular insulin and glucocorticoids, is still required for maintenance of the in vivo-like hepatic phenotype ***(2,14)***. Other commonly used additives, such as serum ***(15)*** and L-proline ***(20)***, can be omitted from collagen gel cultures.

2. The size of the Petri dishes used depends mainly on the aim of the experiment. We routinely use 15- and 10-cm diameter Petri dishes for the extraction of nucleic acids and protein, respectively. In view of performing immunocytochemistry, the hepatocytes are cultivated on a sterilized cover slip in a 35-mm diameter Petri dish. Alternatively, commercially available dishes provided with a cover slip-sealed well can be used (e.g., P35G-0-10-C; MatTek).
3. When fluorescence-based assays are intended, we recommend the use of phenol red–free media (e.g., DMEM 12-917; Bio-Whittaker) to reduce background noise. However, one should keep in mind that the autofluorescence of collagen remains a major cause of background fluorescence.
4. The sterility of the media is checked by adding 1 mL of the prepared medium to 25 mL of autoclaved thioglycollate medium and incubating this mixture at 37°C. After 2 d the potential contamination of the thioglycollate medium is investigated.
5. Although some research groups have focused on drug metabolism in collagen gel cultures of hepatocytes ***(10–13)***, the inter- and intralaboratory reproducibilities of the results obtained in these culture models are not described in the literature.
6. The variables mentioned in Chapter 27 (**Note 4** of this chapter) probably also hold for collagen gel cultures of hepatocytes. To date, however, supporting data are not available.
7. Usually nine rat tails are used and a collagen concentration of about 3 mg/mL is obtained (minimum of 1.7 mg/mL).
8. The collagen type I gel used for immobilization and sandwich cultures is a hydrogel. The process of gel formation is a crucial step in order to obtain a solid gel. Therefore, care must be taken not to disturb this process while transferring freshly coated sandwich cultures or freshly prepared immobilization cultures to the incubator. Moreover, further manipulation of these cultures has to be done with care, because any mechanical stress will cause the gel to detach.
9. From our own experience ***(12,13,16,17,20–22)*** some practical constraints can be identified:
   a. Collagen gel cultures are highly susceptible to bacterial and fungal infections. Therefore, we routinely add a mixture of broad- and narrow-spectrum antibiotics, as well as a wide-spectrum antifungal agent to the culture medium.
   b. The thickness of the collagen layer can be an impediment when using confocal microscopy–based assays. To partially overcome this problem, a water immersion lens can be used with a longer working distance (e.g., Nikon PlanApo 60x NA 1.2).
   c. The exposure of hepatocytes to drugs or substrates is limited owing to the penetration rate through the collagen gel. On this particular topic almost no data are available in the literature ***(23)***.
   d. Normalization of biochemical and metabolic parameters may be problematic. The expression of enzymatic activities vs cytosolic proteins is not possible without enzymatic digestion of the collagen present (*see* **Note 10**). The collagen entraps culture medium proteins, and these cannot be washed out completely. By contrast, the expression of results vs microsomal proteins poses no problems. The expression of enzymatic activities vs the DNA content of the cells is not valid. The DNA remains entrapped in the collagen gel and dying or dead cells show a DNA content identical to the one measured for viable cells.
   e. During the initiation of cultures (especially for immobilization cultures) and as a function of culture time (for both culture models), dying or dead cells can become entrapped in the collagen gel. Not only can their protein and DNA content cause erroneous results, but the leaked enzymes can also damage neighboring cells.

   f. Coculture of hepatocytes with rat liver epithelial cells (RLECs) of primitive biliary origin (as described in the Chapter 27) in both collagen gel models is not successful because confluent layers cannot be reached and the collagen environment seems to be deleterious for RLECs.

10. To obtain access to the hepatocytes for metabolic or toxicity studies, enzymatic digestion of the collagen gel is often necessary. For this purpose, we usually perform the following procedure *(12)*:
    a. Mix 5 parts of $Ca^{2+}$-containing Krebs-Henselit buffer (KHB) with 15 parts of $Ca^{2+}$-free KHB (as described in Chapter 26, **Subheading 2., items 6** and **7**), and gas this mixture for 15 min with carbogen (95% $O_2$ and 5% $CO_2$).
    b. Dissolve 11,500 digestion units of collagenase type I (Clostridiopeptidase A) in 100 mL of the gassed $Ca^{2+}$-containing KHB/KHB mixture.
    c. Remove the medium from the Petri dish and wash twice with ice-cold phosphate-buffered saline (PBS).
    d. Add 2, 4, 11, and 25 mL of the collagenase type I solution to each of the 35-mm and 6-, 10-, and 15-cm diameter Petri dishes, respectively.
    e. Place the Petri dishes in an incubator for 45–60 min (longer incubation times will damage the hepatocytes).
    f. Gently scrape the cells and transfer the cell suspension to a 50-mL centrifuge tube on ice.
    g. Centrifuge at 210*g* for 2 min at 4°C.
    h. Gently remove the supernatant and wash the pellet twice with ice-cold PBS. The resulting pellet can be used for further experiments.

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# 29

## Hepatocytes in Suspension

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**Summary**

Isolated hepatocytes are a physiologically relevant in vitro model exhibiting intact subcellular organelles, xenobiotic transport, and integrated phase I and phase II biotransformation. They represent the "gold standard" for investigating xenobiotic biotransformation and metabolic bioactivation. When used in suspension, they provide an easy-to-handle and relatively cheap in vitro system that can be used for up to 4 h. The use of animal- and human-derived hepatocytes allows interspecies comparisons of metabolic properties. In contrast with microsomes, which are easily prepared from human liver tissue and can be stored in liquid nitrogen with minimal loss of functionality, cryopreservation of isolated human hepatocytes has been shown to be more difficult: after thawing losses of cell viability and biotransformation capacity occur. We provide general recommendations for the appropriate use of hepatocytes in suspension for pharmaco-toxicological studies. We also provide protocols for the cryopreservation of freshly isolated hepatocytes and their handling on thawing.

**Key Words**: Hepatocytes; freshly isolated; cryopreserved; suspension culture; in vitro biotransformation; in vitro hepatotoxicity.

## 1. Introduction

In the early drug discovery stage, hepatocyte and liver microsomal suspensions have been increasingly applied for the metabolic characterization of new chemical entities, usually with the intention of synthesizing analogs with optimized efficacy and/or safety profiles ***(1–4)***. These suspensions are used to gain information on in vivo metabolic clearance, metabolite patterns, and interspecies differences in biotransformation ***(3–5)***. In addition, reaction phenotyping can predict drug–drug interactions ***(6)*** or polymorphic biotransformation ***(3,5,6)***. In general, microsomes are less suitable for clearance prediction than hepatocytes in suspension culture. Reasons are the lack of cytosolic biotransformation systems ***(7)***, end-product inhibition phenomena ***(8)***, the usually nonphysiological levels of added cofactors, and an unnatural orientation of

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biotransformation enzymes (e.g., uridine disphosphate [UDP]-glucuronosyl transferase activation requires the addition of a detergent) *(9)*. Microsomes are especially helpful in the evaluation of compounds that are essentially biotransformed by cytochrome P450 isoenzymes *(7)*. Isolated hepatocytes, by contrast, are a physiologically relevant model with intact subcellular organelles. They are capable of producing their own cofactors at physiological levels and contain all the integrated biotransformation pathways found in the liver in vivo *(10)*. Their intact plasma membrane and xenobiotic transport systems allow differences in plasma and hepatocyte concentrations of xenobiotics, as observed in vivo, to be accounted for *(6,11)*. Moreover, because specific liver functions are expressed at physiological levels, hepatocyte suspensions represent the most suitable system for acute hepatotoxicity testing of compounds. Discrimination between basal cytotoxicity of a compound and specific hepatic toxicity and/or biotransformation-mediated toxicity is possible *(6)*. Because hepatocytes in suspension survive for about 4–6 h, the model is not suitable for long-term biotransformation and toxicity studies or when pretreatment of the cells over an extended period of time is necessary *(3,10)*.

As an alternative to the immediate use of freshly isolated hepatocytes, cells in suspension may be cryopreserved and stored in liquid nitrogen for later use. Considering the limited availability of fresh human livers for research purposes and the ease of using precharacterized cryopreserved hepatocytes at any time, cryopreservation enhances the practical utility of human hepatocytes. Several investigators have reported successful cryopreservation *(1,12–16)*. Cryopreserved hepatocytes have been shown to retain most hepatocellular functions, including active xenobiotic biotransformation and transport. Hepatocyte yields after thawing, however, may vary among species and according to the methodology used *(1,12–15)*. The cryopreservation technique described here is based on the work of Alexandre et al. *(13)* and Chesné et al. *(16)*.

## 2. Materials

### *2.1. Freshly Isolated Hepatocytes in Suspension*

1. Incubation medium: a balanced salt solution supplemented with a bicarbonate and/or an organic buffer such as HEPES, or a complex culture medium.
   a. $Ca^{2+}$-containing Krebs-Henseleit buffer, pH 7.4: 210 mL of 1.294% (w/v) $NaHCO_3$ saturated for 60 min with carbogen (95% air and 5% $CO_2$), 1000 mL of 0.9% (w/v) NaCl, 40 mL of 1.15% (w/v) KCl, 10 mL of 2.1% (w/v) $KH_2PO_4$, and 10 mL of 3.8% (w/v) $MgSO_4 \cdot 7H_2O$ in dd$H_2O$. Sterilize the buffer by passing through a 0.22-µm filter; it can be stored at 4°C for 6 mo. For hepatocyte incubations, this buffer is often supplemented with 10–25 m*M* HEPES and 10 m*M* D-glucose.
   b. HEPES buffer, pH 7.65: 0.800% (w/v) NaCl, 0.010% (w/v) $Na_2HPO_4 \cdot 12H_2O$, 0.020% (w/v) KCl, and 0.038% (w/v) HEPES in dd$H_2O$. Sterilize the buffer over a 0.22-µm filter; it can be stored at 4°C for 6 mo.
   c. Williams' E medium.
2. Stop reagents:
   a. 1.823% (w/v) HCl, 5% (w/v) $HClO_4$ (final concentration).
   b. Acetonitrile, methanol, dimethyl sulfoxide (DMSO).
3. Incubation vials (glass or plastic) or microtiter plates.

4. Shaking water bath or rotation incubator at 37°C.
5. Sonicator.
6. Sterile disposable screw-capped plastic centrifuge tubes.
7. Sterile volumetric pipets.
8. Pipettor.
9. Sterile pasteur pipets.
10. Vacuum aspirator.
11. Liquid nitrogen.

### 2.2. Cryopreserved Hepatocytes

#### 2.2.1. Cryopreservation of Freshly Isolated Hepatocytes in Suspension

1. Cryopreservation medium: Dulbecco's modified Eagle's medium (DMEM) containing 2.5% (w/v) bovine serum albumin fraction V, 20% (v/v) fetal bovine serum (FBS), 2.0% (w/v) polyvinylpyrrolidone, and 10% (v/v) DMSO; sterilize by passing through a 0.22-μm filter and prepare ex tempore.
2. Isopropanol progressive-freezing container.
3. Liquid nitrogen storage unit.
4. Sterile cryogenic Nunc tubes.
5. Sterile disposable screw-capped plastic centrifuge tubes.
6. Sterile volumetric pipets.
7. Pipettor.
8. Sterile pasteur pipets.
9. Vacuum aspirator.

#### 2.2.2. Thawing of Cryopreserved Hepatocytes

1. DMEM supplemented with 10% (v/v) FBS; prepare ex tempore under sterile conditions.
2. Hank's balanced salt solution (HBSS), pH 7.4: 0.0185% (w/v) $CaCl_2$, 0.04% (w/v) KCl, 0.006% (w/v) $K_2HPO_4$, 0.01% (w/v) $MgCl_2{\cdot}6H_2O$, 0.01% (w/v) $MgSO_4{\cdot}7H_2O$, 0.8% (w/v) NaCl, 0.035% (w/v) $NaHCO_3$, 0.0048% (w/v) $Na_2HPO_4$, and 0.1% (w/v) D-glucose in dd$H_2O$. Sterilize the solution by passing through a 0.22-μm filter; it can be stored at 4°C for 6 mo.
3. HBSS containing 20% (v/v) Percoll; prepare ex tempore under sterile conditions.
4. Water bath at 37°C.
5. Sterile disposable screw-capped plastic centrifuge tubes.
6. Sterile volumetric pipets.
7. Pipettor.
8. Sterile pasteur pipets.
9. Vacuum aspirator.

## 3. Methods

### 3.1. Freshly Isolated Hepatocytes in Suspension

General recommendations on experimental conditions for the use of hepatocyte suspensions for xenobiotic biotransformation or toxicity studies are presented in **Table 1**. The incubation with a test substance proceeds as follows:

1. After determining cell yield and viability (*see* **Subheading 3.5.**, Chapter 26; *see* also **Note 1** here), centrifuge the cell suspension for 1 min at 63*g* (room temperature).
2. Discard the supernatant fraction by aspirating with a vacuum aspirator.

**Table 1**
**Recommended Experimental Conditions for Xenobiotic Biotransformation and Toxicity Studies in Hepatocyte Suspension Cultures (Own Experience [17–20])**

| Experimental condition | Recommendation | Motivation |
|---|---|---|
| Incubation time and medium | ≤3 h: balanced salt solution<br>≤6 h: cell culture medium | Cell viability, purpose of study, ease of operation |
| Hepatocyte concentration | 1–5 × $10^6$ viable cells/mL | Good metabolic capacity, low cell aggregation, good cell viability |
| Incubation vial | 1. Disposible glass centrifuge tubes (≤2 h)<br>2. Plastic recipients:<br>2a. Microtiter plates<br>2b. Plastic flasks or Petri dishes | 1. Resistance to acids and organic solvents<br>2. Lower cell adherence<br>2a. Small volumes<br>2b. Larger volumes and longer incubations |
| Incubation volume | 50–100 µL in 96-well plate<br>250 µL in 24-well plate<br>≤1.0 mL in 5.0-mL vial | Adequate oxygenation |
| Incubation conditions | 37°C, 95% relative humidity; 95% air/5% $CO_2$<br>Gentle shaking (90 rpm) | Physiological conditions<br>Inhibition of cell aggregation and adherence |

3. Add a sufficient volume of incubation medium (*see* **Notes 2** and **3**) to bring the concentration to 1–5 × $10^6$ viable cells/mL, and gently resuspend the cell pellet.
4. Preincubate the cell suspension at 37°C for 10 min.
5. Start the incubation by transferring the preincubated cell suspension to an equal volume of test substance solution in incubation medium at room temperature (*see* **Notes 4** and **5**).
6. Incubate the vials or plates at 37°C in a shaking water bath or a rotation incubator. In the case of bicarbonate buffers, gas the suspensions regularly (when using a water bath, gas at least every hour or after opening a vial) or continuously (in the case of an incubator) with carbogen (95% air/5% $CO_2$).
7. Stop the incubation by placing the cell suspensions at or below –80°C or by adding an equal volume of ice-cold stop reagent. In the latter case, sonicate the samples on ice (two 30-s pulses), centrifuge at 2000*g* for 30 min (4°C), and store the supernatant at –80°C (*see* **Notes 6–8**).

## *3.2. Cryopreserved Hepatocytes*

### *3.2.1. Cryopreservation of Freshly Isolated Hepatocytes in Suspension*

1. Centrifuge the freshly isolated hepatocytes for 1 min at 63*g* (room temperature).
2. Discard the supernatant fraction by aspirating with a vacuum aspirator.
3. Add a sufficient volume of ice-cold cryopreservation medium to bring the concentration to 10 × $10^6$ viable cells/mL, and gently resuspend the cell pellet.

4. Divide the homogeneous mixture into aliquots in sterile cryogenic tubes (2 mL) on ice.
5. Within 5 min, cryopreserve the hepatocytes in an isopropanol progressive-freezing container. Place the container at –80°C for 18 h and decrease the temperature at a rate of 1°C/min (*see* **Note 9**).
6. Transfer the vials to a liquid nitrogen storage unit, where they can be stored for several years (*see* **Note 10**).

### *3.2.2. Thawing of Cryopreserved Hepatocytes*

1. Remove one or more vials of cryopreserved hepatocytes from the liquid nitrogen storage unit.
2. Place in a water bath at 37°C until the sample is partially thawed (but not warm) and can be poured from the vial.
3. Gently pour the thawed hepatocyte suspension into a sterile centrifuge tube containing 10 mL of DMEM supplemented with 10% (v/v) FBS (room temperature).
4. Centrifuge for 5 min at 63*g*.
5. Aspirate the supernatant and discard it.
6. Resuspend the cells in 2 mL of HBSS. If several vials were thawed, combine all remaining cells into one tube.
7. Determine the cell concentration and viability (*see* **Subheading 3.5.** in Chapter 26).
8. If cell viability is <60%, an additional Percoll purification needs to be performed (*see* **Notes 11–13**):
   a. Centrifuge the thawed suspension for 2 min at 63*g*.
   b. Discard the supernatant.
   c. Resuspend the cells in HBSS containing 20% (v/v) Percoll (10 mL per $20 \times 10^6$ hepatocytes, room temperature).
   d. Centrifuge for 10 min at 88*g*.
   e. Remove the supernatant.
   f. Resuspend the cells in HBSS (same volume as in **step c**).
   g. Centrifuge for 5 min at 75*g*.
   h. Resuspend the pellet in HBSS (same volume as in **step c**).
   i. Determine the remaining cell number and the viability (*see* **Subheading 3.5.** Chapter 26).
9. Perform incubations as described previously for freshly isolated hepatocytes in **Subheading 3.1.** (*see* **Notes 14–17**).

## 4. Notes

1. By measuring cell viability from the beginning until the end of the suspension culture, information can be gathered on the quality of the hepatocytes. Only freshly isolated hepatocytes exhibiting more than 80% initial viability are acceptable for further experiments ***(3)***.
2. A variety of buffered saline solutions have been used for culturing hepatocytes in suspension. However, little comparative work has been performed. Phosphate buffers are relatively weak and precipitate most polyvalent cations. Bicarbonate buffers require a constant 5% $CO_2$ atmosphere, which is particularly inconvenient when incubation vials are used and samples are taken frequently. In that case, organic buffers such as HEPES and tricine may be preferable ***(17,21,22)***. However, the metabolic pattern observed in these systems may be different from that of a bicarbonate-buffered medium, to the extent that bicarbonate is involved in certain metabolic pathways ***(17)***. The presence of extracellular sulfate or L-cysteine and D-glucose stimulates the synthesis of sulfotransferase and

UDP-glucuronosyl transferase cofactors, respectively, and, therefore, affects the respective apparent kinetic parameters (own experience; *[19]*). The addition of essential amino acids to the incubation medium prevents active degradation of cellular proteins and, therefore, xenobiotic biotransformation enzymes *(17)*. Usually, albumin diminishes cell aggregation and potentially protects against mechanical damage *(17)*. Its addition is, however, a complicating factor when a compound is strongly protein bound. In such a case, it is best omitted from the medium *(21)*.

3. In addition to simple buffer systems, nutritive tissue culture media can be used for the incubation of hepatocytes. However, a medium's components can complicate identification of metabolites or cause underestimation of toxicological events *(3,22)*.
4. The concentration of the compound under consideration will depend on the purpose of the experiment, the sensitivity of the analytical method used, the solubility of the compound used in the culture medium, and its toxicological properties. Because both the rate of biotransformation and the type of metabolites formed can vary according to the availability of the compound, the concentration used should be comparable with the relevant unbound concentration in vivo *(7,23)*. In addition, it should be considered that the amount of substrate available for biotransformation can be affected by nonspecific binding to the incubation recipient or by partitioning into the cell membrane lipids *(20,24,25)*.
5. Substrates that are poorly soluble in water can be added to the medium as small amounts of stock solution in an appropriate organic solvent. Because this solvent may affect cell viability and phase I and II biotransformation activities, both untreated and solvent controls should be used. In general, the volume of organic solvent should be kept lower than 0.5% (v/v) in the case of ethanol, methanol, and acetonitrile, and lower than 0.2% (v/v) in the case of DMSO (own experience; *[26,27]*).
6. For metabolic profiling purposes, both intra- and extracellular metabolite contents should be checked. In addition, it is important to identify non-enzymatic degradation products, which can act as substrates for further biotransformation *(20,28)*. Degradation and condensation reactions of the parent compound and its metabolites can be significantly accelerated during acidic extraction and storage in aqueous medium (own experience; *[28]*).
7. Because of the restricted incubation period of 4 h, minor metabolic pathways that contribute to <3% of the total biotransformation and, in particular, minor phase II metabolites can be missed. They can be identified in hepatocyte monolayer cultures, which allow incubations for 24 h or longer *(20)*.
8. Before the start of an experiment, the functionality of the isolated hepatocytes must be checked using external markers, such as the well-known metabolite profile of testosterone or the activity of 7-ethoxycoumarin *O*-deethylase. A correct qualitative and quantitative metabolite pattern and a specific enzymatic activity within predetermined limits should be obtained *(3,23)*.
9. When cryopreserving hepatocytes according to the widely used stepwise freezing protocol described by Rahmani's group *(29,30)*, on average 75% of the cells are lost during freezing, storage, and thawing *(13,31)*. In the case of the progressive-freezing protocol, recovery is on average 40% *(13,32)*.
10. Cryopreserved hepatocytes are thought to have an infinite storage life when maintained in liquid nitrogen, but rigorous studies are lacking. Therefore, cell yield and viability of preparations should be checked over time.
11. To enhance hepatocyte function after thawing, a preliminary culture step before cryopreservation can be used *(33)*.

12. Percoll gradient techniques may lead to the selection of a particular cell population and a low recovery ***(13)***.
13. If the percentage of viability of the cells is not better after Percoll centrifugation than before, purification should be repeated using a higher Percoll concentration (30% [v/v]). By contrast, if no cell pellet is obtained after Percoll centrifugation, centrifugation time and speed must be slightly increased and the Percoll content decreased ***(5)***.
14. Biotransformation enzyme activities in cryopreserved hepatocytes are usually lower than those observed in freshly isolated cells. Nevertheless, the decrease in phase I metabolizing activities is <50% and considered to be acceptable for drug metabolism studies ***(20)***. In general, preservation of phase II biotransformation enzymatic activities is more problematic compared to phase I enzymes. This is not owing to compromised enzyme activities, because the exogenous addition of cofactors such as UDP-glucuronic acid, for glucuronidation, and 3'-phosphoadenosine-5'-phosphosulfate, for sulfation, can restore phase II biotransformation ***(20,34)***.
15. There is an interspecies variability in the resistance of various biotransformation enzymes to cryopreservation ***(5,13)***. Although UDP-glucuronosyl transferase activity remains unaltered by cryopreservation, glutathione-*S*-transferase activity is strongly reduced in cryopreserved human hepatocytes in contrast to cells from rat and mouse. A similar problem exists for sulfotransferase activity in rat ***(5)***. Because of membrane damage, hepatocytes usually lose their cofactor NADPH during cryopreservation, which can explain a decreased reductive metabolism after thawing ***(5)***.
16. Centrilobular hepatocytes are less susceptible to freezing/thawing conditions than their periportal partners ***(13,16,35)***.
17. Routinely, two strategies are applied with cryopreserved human hepatocytes. The first, the "ten to 1" approach, uses a mixture of cells from 10 donors, resulting in an "average" phenotype, and is adequate for quantitative metabolite identification of metabolites and for interspecies comparison. When information about interindividual differences in biotransformation is needed, several batches of characterized donors must be tested, including poor and extensive metabolizers for the most relevant enzymes ***(20)***.

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# 30

## Isolation of Rat Bone Marrow Stem Cells

**Sarah Snykers, Tamara Vanhaecke, and Vera Rogiers**


#### Summary

Stem cell research has become an important field of study for molecular, cellular, and clinical biology as well as pharmaco-toxicology. Indeed, stem cells have a strong proliferative and unlimited self-renewal potential and are multipotent. In vivo as well as in vitro studies have confirmed the differentiation of adult bone marrow stem cells into muscle cells, adipocytes, cardiomyocytes, neuroectodermal cells, osteoblasts, chondroblasts, and so on. Recently, it has been shown that, under appropriate culture conditions, adult bone marrow stem cells may also differentiate into hepatocyte-like cells. Because of their extensive proliferative capacity and pluripotency, adult bone marrow stem cells could serve in the future as an unlimited source of hepatocytes for pharmaco-toxicological research and testing. We describe a protocol for isolation of mononuclear cells from adult rat bone marrow.


**Key Words:** Isolation; adult bone marrow stem cells; mesenchymal stem cells; hepatocytes; in vitro.

### 1. Introduction

Recently, there has been increased interest in the cellular and molecular biology as well as in the clinical application of adult stem cells. Indeed, stem cells have a strong proliferative and unlimited self-renewal potential and are pluripotent ***(1–10)***. Moreover, this interest in adult stem cells has been sparked in part by the numerous ethical dilemmas concerning the use of embryonic stem cells in preclinical and clinical research ***(11)***. The most characterized stem cell compartment is the bone marrow, consisting of two stem cell populations, referred to as hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) ***(12)***. Several in vivo studies have shown that HSCs differentiate into skeletal muscle cells; cardiacmyocytes; pneumocytes; blood cells; vascular endothelium cells; hepatic cells (oval); biliary duct, lung, gut, and skin epithelia; and neuroectodermal cells ***(8,9,13–18)***. MSCs were first described by Friedenstein and colleagues as a population

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of cells isolated from the bone marrow and capable of differentiation into bone, adipocytes, chondrocytes, osteoblasts, osteoprogenitors, skeletal myocytes, tendon and bone marrow stromal cells *(19–25)*.

Reyes et al. *(26,27)* described a population of cells in the human, mouse, and rat postnatal bone marrow that copurified with MSCs. Not only were these cells capable of differentiation into most types of mesodermal cells, but also into cells of neuroectodermal and endodermal (hepatocytes) origin. These cells were initially called mesodermal progenitor cells and later described as multipotent adult progenitor cells (MAPCs).

MAPCs are clonal and have, at the single cell level, an extensive proliferative capacity without senescence (more than 100 population doublings without telomere shortening). Therefore, MSC and, specifically, MAPCs could serve as an unlimited source of hepatocytes for pharmaco-toxicological research *(1,2,7,28)*. Furthermore, these cells can be easily transfected and engrafted across several immunological barriers, making them ideal vehicles for in vivo therapies of genetic or acquired liver disorders and for use in bioartificial liver devices *(1–3,7,10,11,25–42)*.

Large quantities of MAPCs can be obtained following a simple bone marrow aspiration procedure and subsequent selective expansion of the mononuclear bone marrow stem cells, which is mainly based on self-renewal, size, and expression of non-hematopoietic markers. The present protocol specifically elaborates the procedure for isolating these mononuclear cells from rat bone marrow. After 50 population doublings, rat MAPCs have been shown to differentiate under appropriate culture conditions into hepatocyte-like cells both in vitro and in vivo *(2,7,26,27)*. The culture conditions for further maturation of these cells into functional hepatocytes are currently being optimized.

## 2. Materials

1. Alcohol proanalysis (p.a.).
2. Sodium pentobarbital (60 mg/mL): This solution is stable for several months at 4°C.
3. Phosphate-buffered saline (PBS), pH 7.65: 0.28% (w/v) NaCl, 0.02% (w/v) KCl, 0.31% (w/v) $Na_2HPO_4 \cdot 12H_2O$, and 0.02% (w/v) $KH_2PO_4$ in Millipore-quality water. Sterilize the buffer by passing through a 0.22-µm filter; it can be stored at 4°C for 9 mo.
4. Dulbecco's PBS (DPBS), pH 7.2: 0.01325% (w/v) $CaCl_2 \cdot 2H_2O$, 0.01% (w/v) $MgCl_2 \cdot 6H_2O$, 0.02% (w/v) KCl, 0.02% (w/v) $KH_2PO_4$, 0.8% (w/v) NaCl, and 0.15% (w/v) $Na_2HPO_4$ in Millipore-quality water. Sterilize the buffer by passing through a 0.22-µm filter; it can be stored at 4°C for 9 mo.
5. Dulbecco's modified Eagle medium, low glucose (DMEM-LG), containing glucose (1 g/L), glutamine (584 mg/L), sodium pyruvate (110 mg/L), and pyridoxine hydrochloride: this sterile medium can be stored for 6 mo at 4°C.
6. $10^{-2}$ *M* L-Ascorbic acid 2-phosphate sesquimagnesium salt in PBS: this solution is stable for 1 wk at 4°C.
7. 0.25 m*M* Dexamethasone in $ddH_2O$: this solution is stable for 3 mo at –20°C.
8. MCDB-201 (17.7 g/L) in Millipore-quality water, pH 7.2. Sterilize the medium by passing through a 0.22-µm filter; it can be stored at 4°C for 2 wk.
9. Penicillin (20,000 IU/mL) in PBS: this solution is stable for 1 wk at 4°C.
10. Streptomycin (20 mg/mL) in PBS: this solution is stable for 1 wk at 4°C.

11. Earle's balanced salt solution (EBSS) without phenol red: this sterile medium can be stored for 6 mo at 4°C.
12. 100X insulin/transferrin/sodium selenite (ITS) containing insulin (0.5 g/L), transferrin (0.5 g/L), and sodium selenite (0.5 mg/L) in EBSS: this sterile solution can be stored for 12 mo at 4°C.
13. 100X Linoleic acid-albumin (LA-BSA) containing bovine serum albumin (BSA) (100 mg/mL) in DPBS (*see* **item 4**) and 2 mol of linoleic acid/mol of BSA: this sterile solution can be stored for at least 3 mo at 4°C.
14. Fetal bovine serum (FBS): this sterile solution can be stored for up to 6 mo at 4°C or –20°C.
15. Recombinant human platelet-derived growth factor (PDGF-BB) (10 µg/mL) in 4 m*M* HCl containing 0.1% (w/v) BSA: this sterile solution can be stored for 6 mo at –20°C.
16. Recombinant human epidermal growth factor (EGF) (10 µg/mL) in 10 m*M* acetic acid containing 0.1% (w/v) BSA: this sterile solution can be stored for 6 mo at –20°C.
17. Leukemia-inhibitory factor (LIF) ($10^7$ U): this sterile solution can be stored for several months at 4°C.
18. MAPC medium: 60% (v/v) DMEM-LG, 40% (v/v) MCDB–201 (17.7g/L) supplemented with 1X ITS, LA-BSA (1 mg/mL), penicillin (100 IU/mL), streptomycin (100 µg/mL), 0.1 m*M* L-ascorbic acid, 2% (v/v) FBS, 0.05 µ*M* dexamethasone, PDGF-BB (10 ng/mL), EGF (10 ng/mL), and LIF ($10^3$ U) in Millipore-quality water. Sterilize the medium by passing through a 0.22-µm filter; it can be stored at 4°C for 2 wk.
19. Ficoll-Paque (density: 1.077 g/L): this sterile solution can be stored for 6 mo at 4°C.
20. Laminar flow cabinet.
21. Autoclaved dissection material.
22. Aluminium foil.
23. Sterile compresses.
24. Sterile gloves.
25. Three sterile 6-cm Petri dishes.
26. Centrifuge at 4°C.
27. Centrifuge at 20–25°C.
28. Sterile volumetric pipets.
29. Micropipets.
30. Sterile tips.
31. Sterile 15-mL conical tubes.
32. Sterile 2-mL syringes.
33. Sterile 21-gage needles.
34. Hemacytometer.
35. Inverse-phase light microscope.

## 3. Methods

### *3.1. Isolation of Rat Mononuclear Bone Marrow Cells (see Note 1)*

1. Sterilize a laminar flow cabinet with 70% (v/v) alcohol, and place all necessary equipment (aluminium foil, sterile compress, sterile gloves, three sterile 6-cm Petri dishes, sterile volumetric pipets, micropipets, sterile tips, sterile 15-mL conical tubes, sterile 2-mL syringes, sterile 21-gage needles) in the flow cabinet.
2. Use a male Sprague-Dawley rat (4–6 wk old) that has access to water and food ad libitum (*see* **Note 1**).
3. Anesthetize the rat by injecting sodium pentobarbital solution (0.1 mL/100 g of body wt) intraperitoneally.

4. Shave the rat, disinfect the skin with alcohol (70% [v/v]), and transfer the rat to a surgery table in a laminar flow cabinet.
5. Remove the skin of one leg (pars libera membri inferioris), and then gently remove the tissue, fat, and muscles surrounding the tibia and femur to expose the bone (do not cut within the muscle, but try to cut parallel between the muscles and bone).
6. After clearing the bone, cut the bone at the articulatio coxae and talotarsalis. Leave both condyli intact, and keep the femur and tibia connected (*see* **Note 2**).
7. Transfer the isolated bone into a sterile 6-cm Petri dish, filled with 6 mL of cold PBS.
8. Repeat **steps 5–7** for the remaining rat partes liberae membri inferioris.
9. Collect all bones in the same 6-cm Petri dish.
10. Separate the femur and tibia by cutting the bone at the articulatio genus. Leave the condyli of both the tibia and femur intact (*see* **Note 2**).
11. Transfer the separated femur and tibia into a new sterile 6-cm Petri dish filled with 6 mL of cold PBS.
12. Repeat **steps 10** and **11** for the other rat bones, kept in cold PBS.
13. Cut away the condyli of the tibia and femur.
14. Fill a sterile 2-mL syringe with sterile cold MAPC medium and fit a sterile 21-gage needle to it.
15. Flush the bone marrow with the 21-gage needle into a new sterile 6-cm Petri dish filled with 6 mL of cold MAPC medium (*see* **Note 3**).
16. Continue flushing until the bone appears white.
17. Disperse red clumps of cells by passing the cell suspension through the 21-gage needle until no more clumps are seen.
18. Repeat **steps 13–17** for all isolated bones.

### 3.2. Purification of Bone Marrow Cells

1. Transfer the cell suspension in cold MAPC medium to a 15-mL conical tube.
2. Spin for 5 min at 675*g* and 4°C (brake:high).
3. Discard the supernatant carefully.
4. Resuspend the pellet in 6 mL of MAPC medium at room temperature.
5. Pipet 6 mL of Ficoll-Paque (1.077 g/L) in a new sterile 15-mL conical tube, and very carefully layer the homogenized cell suspension on top of it.
6. Centrifuge for 20 min at 675*g* and room temperature (20–25°C) with the brake off (*see* **Note 4**).
7. Remove the MAPC medium layer very carefully, leaving 1 mL on top of the buffy coat (*see* **Note 5**). Or, very carefully remove almost all of the MAPC medium layer, leaving 1 mL on top of the buffy coat (*see* **Note 5**).
8. Draw 10 mL of MAPC medium into a pipet and collect the buffy coat while gently swirling the pipet in the buffy coat layer (*see* **Note 6**).
9. Transfer the cell suspension to a new 15-mL conical tube and homogenize by pipetting up and down.
10. Centrifuge for 5 min at 675*g* and 4°C (brake:high).
11. Discard the supernatant and resuspend the pellet in 10 mL of MAPC medium.

### 3.3. Determination of Number of Viable Cells and Cell Viability (see Note 1)

1. Clean both the hemacytometer and the glass cover slip with alcohol (70% [v/v]).
2. Place the glass cover slip over the groves and semisilvered counting area.

3. Dilute 5 μL of cell suspension to 50 μL with MAPC medium, and mix with 50 μL of Trypan blue.
4. Transfer the cell suspension to the edge of the hemacytometer, and allow the suspension to spread evenly by capillarity.
5. Count the viable (white) and dead (blue) cells in the hemacytometer under a light microscope (10× objective). Usually $4 \times 1$ mm$^2$ areas are counted per chamber.
6. Calculate the cell density and viability as follows:

   Viability (%) = (no. of viable cells × 100)/[(no. of viable cells) + (no. of dead cells)]

   Cell density (cells/mL) = [(no. of viable cells) × 20 × 1000]/0.1
   = (no. of viable cells) × 20 × $10^4$

   in which 20 = the dilution factor, the depth of the hemacytometer = 0.1 mm, the area counted = 1 mm$^2$, therefore volume = 0.1 mm$^3$ = $10^{-4}$ mL. Only cell suspensions with a viability of 90% or greater are used for in vitro purposes.

## 4. Notes

1. To minimize variation among cultures obtained from different bone marrow stem cell isolations, the following parameters should be kept rigorously constant:
   a. With respect to the bone marrow stem cell isolation procedure:
      i. Isolation time.
      ii. Method for removing tissue, fat, and muscles of the tibia and femur.
      iii. Method for separating the condyli of the patella, thigh, and talus joints.
      iv. Type of needle.
      v. Temperature, pH, and composition of the dispersing medium (MAPC medium) for the freshly isolated bone marrow cells (e.g., presence, concentration and type of FBS and BSA).
      vi. Washing and purification procedures of the cell suspension (length of centrifugation time, *g* force, number of washings, temperature, pH and volume of MAPC medium, and density and volume of Ficoll-Paque).
   b. With respect to the cell source:
      i. Strain, age, gender, and health condition of the rats.
      ii. Food composition, feeding schedule, type of bedding, light/dark cycle, and environmental stress factors.
   c. With respect to determination of the number of viable cells:
      i. Counting: this should always be done in the same way in order to plate after every isolation an equal number of cells per well/flask. Even better is that cells are always be counted by the same person.
2. The cell viability depends greatly on the intactness of the condyli of the femur and tibia. The exposure time of bone marrow cells to air should be kept as short as possible.
3. As already mentioned (*see* **Note 2**), for cell viability, it is crucial to minimize the exposure of bone marrow cells to air. Therefore, the tibia and femur, respectively, should be held in the dispersing medium while flushing the cells into the Petri dish.
4. During this centrifugation step, it is highly recommended to work at room temperature, to improve separation based on density. Furthermore, it is crucial to centrifuge without a brake; if not, the interface will be disturbed.
5. Based on differences in density, some cells pellet (red blood cells, cell clusters), whereas others form a buffy coat (mononuclear cells) at the interface of Ficoll-Paque and MAPC medium.

6. Before collecting the buffy coat, it is important first to "coat" the pipet with FBS (present in MAPC medium). If not, the cells will stick/attach to the pipet.

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# 31

## Transfection of Primary Cultures of Rat Hepatocytes

**Mina Edwards, Siew Cheng Wong, Roongsiri Chotpadiwetkul, Despina Smirlis, Ian R. Phillips, and Elizabeth A. Shephard**

### Summary

Five different transfection reagents—calcium phosphate, TransFast™ Transfection Reagent, Superfect™ Transfection Reagent, Effectene™ Transfection Reagent, and Tfx™-20—were compared for their ability to effectively transfect primary cultures of male rat hepatocytes. Hepatocytes were isolated by the collagenase perfusion method and then cultured on Matrigel-coated plates for 24 h before transfection. The cells were transfected with either pGL3-Control or pGL3-Basic plasmids. The efficiency of transfection of each reagent was monitored using the dual luciferase reporter gene assay system. Superfect Transfection Reagent, Effectene Transfection Reagent and Tfx-20 were the most effective for the transfection of primary hepatocytes and gave comparable transfection efficiencies. Calcium phosphate was found to be the least effective transfection reagent and gave the most variable transfection results. Tfx-20 gave the least variable transfection results when different hepatocyte preparations were compared.

**Key Words:** Hepatocytes; transfection; reporter gene assay.

### 1. Introduction

Genes encoding many cytochromes P450 (CYPs) are activated by xenobiotics such as therapeutic drugs and natural products *(1,2)*. Analysis of the factors involved in the regulation of a particular CYP gene by a xenobiotic requires transfection of cells in culture with suitable DNA constructs. The DNA constructs may comprise putative enhancer or repressor sequences attached to an easily assayable reporter gene such as luciferase, β-galactosidase, or chloramphenicol acetyltransferase. Cells can also be cotransfected with an enhancer-reporter construct together with an expression vector containing the cDNA for a transcription factor suspected of interacting with the enhancer/repressor sequence in question *(2)*. The key to obtaining meaningful results from such experiments is to use a cell system in which the gene to be studied is normally activated in response to the particular foreign chemical. For several CYP genes,

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such as *CYP2B1* and *CYP2B2* genes, which are activated by the antiepileptic drug phenobarbital, the most suitable system is primary hepatocytes *(2)*, because immortal cell lines, even if they are derived from a hepatic origin, have often lost the ability to respond to foreign chemicals such as phenobarbital *(3)*. The isolation and culture of primary cells is expensive and the cells have a short life-span. Therefore, it is necessary that the transfection method used for the uptake of DNA by the cells be both rapid and effective.

Here we describe five different protocols that we have used for the transfection of primary rat hepatocyte cultures. The methods differ with respect to the way in which the DNA to be transfected is complexed for its uptake into hepatocytes.

## 2. Materials

### 2.1. Hepatocyte Culture

1. Williams' E medium with $NaHCO_3$ and without L-glutamine and phenol red (Sigma/Aldrich, Poole, UK): store at 4°C.
2. GlutaMAX-I Supplement (Invitrogen, Paisley, UK): this is supplied as a 200 m*M* solution in 0.85% (w/v) NaCl; store at –20°C.
3. Antibiotics: penicillin (200 U/mL), streptomycin (200 µg/mL), and amphotericin (2.5 µg/mL) (Invitrogen). Store at –20°C.
4. Insulin from bovine pancreas (Sigma/Aldrich): prepare a 10 mg/mL stock solution by reconstituting 100 mg of lyophilized powder in 10 mL of sterile deionized distilled water and adding 100 µL of glacial acetic acid. Mix gently until the solution becomes clear. Store at 4°C.
5. 8 m*M* Dexamethasone phosphate (David Bull Laboratories, Warwick, UK): dilute to 1 µ*M* in Williams' E medium; store at 4°C.
6. Dialyzed fetal bovine serum (D-FBS): 10,000 mol wt cutoff (Invitrogen). Store at –20°C.
7. Fully supplemented medium: Williams' E medium containing 2 m*M* GlutaMAX-I, penicillin (200 U/mL), streptomycin (200 µg/mL), amphotericin (2.5 µg/mL), insulin (1.7 µ*M*), dexamethasone (0.1 µ*M*), and 10% (v/v) D-FBS.
8. Permanox plates, 60-mm diameter (Nalge Nunc, Naperville, IL).
9. Matrigel (1 mg/mL) (Becton Dickinson, Stratech Scientific; Luton, UK): store at –20°C.
10. Matrigel-coated Permanox plates (*see* **Note 1** and **Subheading 3.1.**).
11. Hepatocytes isolated from male rat livers as described in Chapter 26: Suspend the hepatocytes at a concentration of $2 \times 10^6$/mL in fully supplemented medium (*see* **Note 2**).

### 2.2. Transfection of Hepatocytes

#### 2.2.1. Transfection Using Calcium Phosphate

1. 250 m*M* $CaCl_2$: $CaCl_2 \cdot 4H_2O$ Suprapur (Merck, Darmstadt, Germany). Filter-sterilize and prepare on the day of the experiment.
2. 2X HeBS: 0.28 *M* NaCl, 10 m*M* KCl, 1.5 m*M* $Na_2HPO_4$, 42 m*M* HEPES, and 2% (w/v) glucose. Adjust to pH 7.1.
3. HeBS-glycerol: 15% (v/v) glycerol in 1X HeBS.
4. Hank's solution: 137 m*M* NaCl, 5.4 m*M* KCl, 0.4 m*M* $MgSO_4$, 0.5 m*M* $MgCl_2$, 0.35 m*M* $Na_2HPO_4$, 0.44 m*M* $KH_2PO_4$, and 2 m*M* HEPES. Adjust to pH 7.4 with NaOH.
5. Supercoiled plasmid DNA (*see* **Note 3**).
6. Falcon tubes (15 mL) (BD BioSciences Clontech, Oxford, UK).

7. Williams' E medium (*see* **Subheading 2.1., item 1**)
8. Fully supplemented medium (*see* **Subheading 2.1., item 7**).

#### 2.2.2. Transfection Using TransFast™ Transfection Reagent

The transfection reagent is supplied in a kit form (Promega UK, Southampton, UK).

1. TransFast Transfection Reagent: store at –20°C (*see* **Note 4**).
2. Nuclease-free water: store at –20°C.
3. Supercoiled plasmid DNA (*see* **Note 3**).
4. Williams' E medium (*see* **Subheading 2.1., item 1**).
5. Fully supplemented medium (*see* **Subheading 2.1., item 7**).
6. 10X phosphate-buffered saline (PBS) (Invitrogen).

#### 2.2.3. Transfection Using Superfect™ Transfection Reagent

The transfection reagent is supplied in a kit form (Qiagen, Crawley, UK).

1. Superfect Transfection Reagent: Store at –20°C.
2. Supercoiled plasmid DNA (*see* **Note 3**).
3. Williams' E medium (*see* **Subheading 2.1., item 1**).
4. Fully supplemented medium (*see* **Subheading 2.1., item 7**).
5. 10X PBS (Invitrogen).

#### 2.2.4. Transfection Using Effectene™ Transfection Reagent

The transfection reagent, enhancer, and buffer EC are supplied in a kit form (Qiagen).

1. Effectene Transfection Reagent (1 mg/mL): store at 4°C.
2. Enhancer (1 mg/mL): store at 4°C.
3. Buffer EC: store at 4°C.
4. Supercoiled plasmid DNA (*see* **Note 3**).
5. Williams' E medium (*see* **Subheading 2.1., item 1**).
6. Fully supplemented medium (*see* **Subheading 2.1., item 7**).
7. 10X PBS (Invitrogen).

#### 2.2.5. Transfection Using Tfx™-20 Reagent

Tfx-20 Transfection Reagent is supplied as a dried lipid film (Promega, Madison, WI).

1. Tfx-20 reagent: store at –20°C. We purchase vials containing 1.6 mg of Tfx-20.
2. Nuclease-free water (supplied with the transfection reagent): store at –20°C.
3. Supercoiled plasmid DNA (*see* **Note 3**).
4. Williams' E medium (*see* **Subheading 2.1., item 1**).
5. Fully supplemented medium (*see* **Subheading 2.1., item 7**).
6. 10X PBS (Invitrogen).

### 2.3. Dual Luciferase Reporter Assay System

The reagents for the reporter gene assay are supplied in a kit form (Qiagen).

#### 2.3.1. Cell Lysis

1. 1X PBS without calcium and magnesium (PAA Laboratories, Linz, Austria) (*see* **Note 5**): filter-sterilize.

2. 5X Passive lysis buffer (PLB): store at –20°C. Before use, dilute to 1X with sterile distilled water. Prepare sufficient working solution for each experiment.
3. Orbital shaking platform, cell scraper.
4. Eppendorf tubes (1.5-mL).

#### 2.3.2. Luciferase Assay

1. Luciferase assay buffer II (LA buffer II): store at –20°C.
2. Luciferase assay substrate: resuspend lyophilized product in 10 mL of LA buffer II. Store in 1-mL aliquots at –20°C, labeled as LAR II (luciferase assay reagent II). Before use, thaw the reagent in a water bath at room temperature and mix well by gentle vortexing.
3. Stop & Glo substrate solvent: store at –20°C.
4. Stop & Glo substrate: prepare a 50X stock solution by diluting in Stop & Glo substrate solvent. Store at –20°C.
5. Stop & Glo buffer.
6. Stop & Glo reagent: in a siliconized, 30-mL Corex tube, dilute Stop & Glo substrate in Stop & Glo buffer to give a 1X solution. Prepare fresh.
7. 1X PBS without calcium and magnesium (PAA Laboratories) (*see* **Note 5**): filter-sterilize.
8. Luminometer, luminometer tubes.

## 3. Methods

### 3.1. Hepatocyte Culture

1. Prepare Matrigel-coated plates. Using a precooled pipet, dilute Matrigel in Williams' E medium to a concentration of 1 mg/mL. Pipet 200 μL onto each 60-mm Permanox plate. Leave the plates at room temprature so that the matrix will gel and use within 2 to 3 h (*see* **Note 6**).
2. Plate 2 mL of cell suspension/coated plate (*see* **Subheading 2.1., item 11**) to give a plating density of 4 to 5 × $10^6$ cells/plate. Swirl the solution gently so that the cell suspension covers the entire plate area.
3. Incubate for 3 h at 37°C in 5% $CO_2$, to allow the cells to adhere to the plate.
4. Discard the medium. Wash the adhered cells two to three times with 3 mL of Williams' E medium.
5. Add 4 mL of fully supplemented medium to each plate and leave for about 20 h at 37°C in 5% $CO_2$. The cells are now ready for transfection and/or chemical treatment (*see* **Note 7**).

### 3.2. Transfection of Hepatocytes

In all cases, transfections were carried out on primary hepatocytes that had been cultured as described under **Subheading 3.1.** for a total of 24 h. A comparison of the effectiveness with which hepatocytes were transfected with each of the reagents is shown in **Table 1**. For the purposes of comparing the different transfection reagents, the same amount of DNA was transfected per plate with each transfection reagent. The pGL3-Control vector served as the positive control for transfection. It contains the SV40 promoter upstream of the firefly (*Photinis pyralis*) luciferase reporter gene and an SV40 enhancer downstream of it. The pGL3-Basic vector also contains the firefly luciferase reporter gene. This plasmid, however, contains no promoter or enhancer to drive the reporter gene and thus serves as a negative control. The pRL-TK plasmid contains the Renilla (*Renilla reniformis*) luciferase reporter gene driven by the herpes

**Table 1**
**Reporter Gene Activity in Primary Cultures of Rat Hepatocytes[a]**

| Plasmid | Transfection reagent | Relative Light Units |
|---|---|---|
| pGL3-Basic | $CaPO_4$ | 1.04 ± 0.28 |
| pGL3-Control | $CaPO_4$ | 190.33 ± 31.79 |
| pGL3-Basic | TransFast | 1.03 ± 0.27 |
| pGL3-Control | TransFast | 226.53 ± 118.92 |
| pGL3-Basic | Superfect | 0[b] |
| pGL3-Control | Superfect | 800[b] |
| pGL3-Basic | Effectene | 0.53 ± 0.91 |
| pGL3-Control | Effectene | 1041.14 ± 64.78 |
| pGL3-Basic | Tfx-20 | 1.48 ± 0.12 |
| pGL3-Control | Tfx-20 | 862.20 ± 12.25 |

[a] Transfections using calcium phosphate ($CaPO_4$) and TransFast were carried out on the same batch of hepatocytes, while transfections using Superfect and Effectene were performed at the same time on another batch of hepatocytes. A third batch of hepatocytes was transfected with Tfx-20.

[b] $n = 2$.

simplex virus thymidine kinase gene promoter. This plasmid is used to normalize transfection efficiencies among different culture plates.

### *3.2.1. Transfection Using Calcium Phosphate*

The calcium phosphate transfection method involves the precipitation of DNA with calcium chloride in a PBS solution. The DNA precipitate is taken into the cells by endocytosis or phagocytosis. This method is the least expensive method for the transfection of cells, but it is the one that gave the greatest variation both between replicate plates from the same hepatocyte preparation and between hepatocytes prepared on different days. The method used is based on that described by Gaunitz et al. ***(4)***.

1. Dilute plasmid DNA in 250 m*M* $CaCl_2$ (*see* **Note 8**).
2. For each 60-mm-diameter culture dish, 343 µL of $CaPO_4$/DNA precipitate containing 4.1 µg of DNA is required (*see* **Note 9**). Prepare this in a Falcon tube by adding dropwise, using a glass Pasteur pipet, 171.5 µL of 250 m*M* $CaCl_2$ containing 4.1 µg of DNA to 171.5 µL of 2X HeBS. During the addition, mix the solution by bubbling air into the tube using a second glass Pasteur pipet attached to a mechanical pipet aid (*see* **Note 10**). The addition of DNA should take at least 1 min. Vortex the precipitate at maximum speed for 2–3 s and incubate at room temperature for 25–30 min. Check the precipitate under a microscope to ensure that the prepared precipitate is one of fine particles and that no clumps are visible.
3. Just before transfection, remove the culture medium from the cells and add 2.57 mL of fully supplemented medium without D-FBS (*see* **Note 11**). Mix the $CaPO_4$/DNA precipitate by pipetting up and down with a 1-mL Gilson pipet and add 343 µL to the medium. Swirl the medium gently during addition of the $CaPO_4$/DNA mixture, and do not leave at room temperature for longer than 6 min (*see* **Note 12**).

4. Leave the precipitate on the cells for 5 h at 37°C under 5% $CO_2$.
5. Wash the cells twice with Hank's solution. Add 860 μL of HeBS-glycerol to each plate, and incubate for exactly 2 min at room temperature. Remove the HeBS-glycerol promptly, and wash the cells twice with Hank's solution and then once with Williams' E medium.
6. Add 3 mL of fully supplemented medium without FBS and culture for 24 h.
7. Wash the cells once with Williams' E medium.
8. Add 3 mL of fully supplemented medium without FBS (*see* **Note 11**).
9. Culture the cells for an additional 24 h.
10. Lyse the cells and monitor reporter gene activity as described in **Subheading 3.3.**

### 3.2.2. Transfection Using TransFast Transfection Reagent

TransFast Transfection Reagent is a mixture of a neutral lipid, L-dioleoyl phosphatidylethanolamine, and a synthetic cationic lipid, (+)-*N*,*N*[bis(2-hydroxyethyl)]-*N*-methyl-*N*-][2,3-di(tetradecanoyloxy)propyl]ammonium iodide. It forms a liposome/nucleic acid complex that is thought to enter a cell via endocytosis or by fusion of the lipid moieties with the plasma membrane. Delivery of DNA into cells is via a liposome-mediated process. The procedure described is adapted from that recommended by the manufacturer *(5)*.

1. The day before transfection, resuspend the TransFast reagent in 400 μL of nuclease-free water by vortexing for 10 s at room temperature. Store frozen at –20°C.
2. For each 60-mm culture dish, prepare a mix of 4.1 μg of DNA (*see* **Note 9**) with 2 mL of Williams' E medium (*see* **Note 13**). To this add 24.4 μL of TransFast reagent and vortex briefly. Incubate for 10–15 min at room temperature.
3. Remove the medium from the cells and wash once with Williams' E medium.
4. After the incubation period is complete, vortex the DNA mixture and pour onto the cells.
5. Incubate the cells in a 37°C incubator with 5% $CO_2$ for 1 h.
6. Gently layer 4 mL of fully supplemented medium onto the cells and incubate for another 24 h.
7. Wash the cells once with Williams' E medium.
8. Add 4 mL of fully supplemented culture medium.
9. Culture the cells for an additional 24 h.
10. Lyse the cells and monitor reporter gene activity as described in **Subheading 3.3.**

### 3.2.3. Transfection Using Superfect Transfection Reagent

Superfect Transfection Reagent is a highly branched polycation of defined shape and diameter that packages DNA into compact structures *(6)*. The particles formed have a net positive charge, thus allowing them to bind with negatively charged receptors on the cell surface. The procedure used is adapted from that described by the manufacturer *(7)*.

1. The day before transfection, for each 60-mm culture plate, dilute 4.1 μg of plasmid DNA with Williams' E medium to a total volume of 150 μL and store at 4°C.
2. On the day of transfection, add 30 μL of Superfect Transfection Reagent to the 150 μL of DNA solution (*see* **Note 9**) and mix by vortexing for 10 s. Incubate the mixture for 5–10 min at room temperature to allow formation of the complex between DNA and the transfection reagent.

3. Remove the culture medium from the cells and wash once with Williams' E medium.
4. Add 1 mL of fully supplemented medium to the DNA complex, and mix the contents by pipetting the solution up and down twice. Immediately pipet the entire volume onto the cells.
5. Incubate the cells in a 37°C incubator in 5% $CO_2$ for 2–3 h.
6. Remove the medium and wash the cells with Williams' E medium.
7. Add 4 mL of fully supplemented medium and incubate at 37°C with 5% $CO_2$ for 24 h.
8. Wash the cells once with Williams' E medium and add 4 mL of fully supplemented culture medium.
9. Culture the cells for an additional 24 h.
10. Lyse the cells and monitor reporter gene activity as described in **Subheading 3.3.**

### 3.2.4. Transfection Using Effectene Transfection Reagent

Effectene is a nonliposomal lipid ***(8)***. The method described is adapted from that described by the manufacturer.

1. Dilute 4.1 µg of DNA (*see* **Note 9**) in buffer EC to a total volume of 150 µL. Add 8 µL of enhancer.
2. Mix solution by vortexing briefly. Incubate at room temperature for 2–5 min.
3. Add 25 µL of Effectene Transfection Reagent and vortex for 10 s. Incubate at room temperature for 5–10 min.
4. Wash the plates once with Williams' E medium.
5. Add 4 mL of fully supplemented medium/plate.
6. Add 1 mL of fully supplemented medium to the DNA solution. Mix by pipetting up and down twice; then, using the pipet, immediately add the DNA solution dropwise to the plate of cells.
7. Incubate the cells at 37°C, 5% $CO_2$ for 24 h.
8. Wash the cells once with Williams' E medium.
9. Add 5 mL of fully supplemented medium.
10. Culture the cells for an additional 24 h.
11. Lyse the cells and monitor reporter gene activity as described in **Subheading 3.3.**

### 3.2.5. Transfection Using Tfx-20 Reagent

Tfx-20 reagent is a mixture of a synthetic, cationic lipid molecule (*N,N,N′,N′*-tetramethyl-*N,N′*-bis[2-hydroxyethyl]–2,3-di[oleoyloxy]-1,4-butanediammonium iodide) and the neutral lipid L-dioleoyl phosphatidylethanolamine. The transfer of DNA into cells is aided by the association of DNA with lipid vesicles that fuse with the cell membrane. Cells grown in serum can be transfected with the Tfx range of reagents, and, thus, their viability is not compromised. The method described is adapted from that recommended by the manufacturer ***(9)*** and has been successfully used to transfect primary cultures of rat hepatocytes ***(2,3)***.

1. The day before transfection set up a water bath at 65°C.
2. Warm a vial containing Tfx-20 to room temperature for about 10–15 min.
3. Thaw nuclease-free water.
4. Remove the cap and septum from the vial containing Tfx-20 and add 400 µL of water. The final concentration of the cationic lipid component will be 1 m*M*.

5. Replace the septum and immediately vortex the vial vigorously for 10–15 s. Be sure to hold a finger firmly on the septum while vortexing.
6. Cover the septum with Nescofilm and incubate the vial at 65°C for only 1 min. Make sure that the water level is above the liquid in the vial, and hold the septum firmly onto the vial to keep it from popping off.
7. Vortex the vial for about 10–15 s and then store at –20°C.
8. On the day of transfection, thaw the hydrated Tfx-20 at room temperature and vortex the solution. If liquid has accumulated at the top of the vial, centrifuge at 300*g* for 3 min to collect the liquid down into the vial.
9. In a 5-mL sterile tube add 2 mL of prewarmed Williams' E medium (*see* **Subheading 2.1., item 1**).
10. To each tube add the appropriate amount of DNA (*see* **Note 9**) (the total amount should not exceed 5 µg), and vortex for 10–15 s.
11. Add 3 µL of Tfx-20 reagent/µg of DNA to each tube (charge ratio of 2:1, reagent:DNA; *see* **Note 14**), and vortex immediately for 15 s. The unused Tfx-20 reagent can be stored at –20°C (*see* **Note 15**).
12. Incubate the Tfx-20 /DNA mixture at room temperature for 15 min.
13. Remove the medium from the hepatocytes.
14. Briefly vortex the Tfx-20/DNA mixture and add 2 mL to each 60-mm plate.
15. Incubate the cells for 1 h at 37°C in 5% $CO_2$.
16. While the cells are incubating, warm fully supplemented medium (*see* **Subheading 2.1., item 7**).
17. After 1 h, without removing the transfection reagent, gently overlay the cells with 4 mL of fully supplemented medium.
18. Culture the cells for an additional 24 h.
19. Lyse the cells and monitor reporter gene activity as described in **Subheading 3.3.**

### 3.3. Dual Luciferase Reporter Assay System

For each transfection method tested, multiple plates of hepatocyte cultures were transfected with either pGL3-Control, as the positive control, or pGL3-Basic, as the negative control (**Table 1**).

#### 3.3.1. Cell Lysis

1. Pour off the medium. Add 1 mL of 1X PBS (without calcium and magnesium) to a culture dish. Swirl the plate and remove the PBS. Repeat. Ensure that all the PBS is removed.
2. Add 400 µL of 1X PLB/plate. Place the plates on an orbital shaking platform for 15 min at room temperature. Examine the cells under a microscope. If required, leave the cells at room temperature until cell lysis is complete.
3. Scrape the cells off the plate and transfer the contents of each plate into a separate 1.5-mL Eppendorf tube.
4. If the samples are to be assayed immediately, first place the tubes at –20°C to freeze and then thaw (*see* **Note 16**).
5. Centrifuge for 30 s at maximum speed in a refrigerated microcentrifuge.

#### 3.3.2. Reporter Gene Assay

1. Dispense 100 µL of LAR II into the required number of luminometer tubes.
2. Working with one sample at a time, add 20 µL of lysate to 100 µL of LAR II. Mix the contents by pipetting up and down two or three times.

3. Place the tube in a luminometer and take the first luminescence reading (*see* **Note 17**).
4. Remove the tube and add 100 µL of 1X Stop & Glo reagent. Mix the contents by pipetting up and down two or three times.
5. Place the tube in the luminometer again and take a second luminescence reading (*see* **Note 17**).

### 3.4. Conclusion

Superfect Transfection Reagent, Effectene Transfection Reagent, and Tfx-20 were the most effective for the transfection of primary hepatocytes and gave comparable transfection efficiencies (**Table 1**). Calcium phosphate was found to be the least effective transfection reagent and gave the most variable transfection results. Tfx-20 gave the least variable transfection results when different hepatocyte preparations were compared and is the reagent used in the experiments described in **refs.** ***2*** and ***3***.

## 4. Notes

1. Plates must be freshly coated with Matrigel. Do not be tempted to store precoated plates. It is best to freeze Matrigel in aliquots suitable for a single experiment. Do not freeze-thaw diluted or undiluted Matrigel more than twice.
2. The viability of the hepatocyte cell population must be >75%. Viability is assessed by Trypan blue exclusion.
3. Plasmid DNA was prepared using the Maxi Qiagen plasmid kit according to the manufacturer's instructions. The quality of the DNA was assessed by agarose gel electrophoresis. For reproducibility among experiments, and to ensure good transfection, at least 90% of the DNA should be in the supercoiled form. To compare the transfection methods, we used pGL3-Control plasmid to monitor expression of the luciferase reporter gene. As a negative control, pGL3-Basic plasmid was used (Promega UK).
4. Dried Transfast Reagent is stable for 6 mo, whereas the hydrated reagent is stable for up to 2 mo, at –20°C.
5. The PBS used for cell lysis must be calcium and magnesium free to avoid inhibition of luciferase activity.
6. Prechill all plasticware, including Permanox plates, before plating with Matrigel. Keep everything on ice, including the solutions and plasticware used in steps carried out inside a hood. Do not store Matrigel-coated plates. Prepare the plates on the morning of the experiment, and while the Matrigel is setting, proceed with the isolation of hepatocytes.
7. Cells are now at the stage in which a xenobiotic, or other test chemical, can be added. For example, we add phenobarbital at a final concentration of 0.1 m*M*.
8. The DNA can be diluted the day before the transfection and stored at 4°C. However, the precipitate must be prepared 30 min before transfection of the cells.
9. Although we used 4.1 µg of DNA for the purpose of comparing the different transfection methods that we describe, up to 5 µg of DNA can be transfected without harming the cells. If two or more plasmids are to be cotransfected, the total amount of DNA should not exceed 5 µg. The Renilla and Firefly reporter plasmids were used in a ratio of 1:10, respectively, in all the transfection protocols described.
10. Hold the second pipet such that the air will blow into the bottom of the tube.
11. We have also carried out this method of transfection when D-FBS was present. No difference in efficiency of transfection was observed.
12. This is the time period recommended by Gaunitz et al. ***(4)***.

13. The mixture used here gives a charge ratio of TransFast reagent to DNA of 2:1. For a ratio of 1:1, add 3 μL of TransFast to 1 μg of DNA. It is best to carry out a preliminary experiment on the plasmids to be used, to determine the optimum ratio of DNA to transfection reagent.
14. We have found that the optimum charge ratio of Tfx-20 reagent to DNA is 2:1.
15. Dried Tfx-20 lipid film is stable for 6 mo at –20°C, whereas the hydrated form of the reagent is stable for about 8 wk. The reagent is not affected by up to 10 freeze-thaw cycles.
16. To achieve consistent results, and to ensure cell lysis, we have found it necessary to subject the lysates to one freeze-thaw cycle prior to assay of the luciferase reporter genes. It is convenient to store the lysates overnight at –20°C and assay the following day. However, this freeze-thaw cycle was not found to be necessary when cells were transfected with other reporter genes such as the chloramphenicol acetyltransferase gene. Lysates may be kept at –70°C for long-term storage.
17. The first luminometer reading measures the activity of Firefly luciferase. Following the addition of the Stop & Glo reagent a second reading is taken. This measures the activity of Renilla luciferase. Results are expressed as the ratio of Firefly:Renilla luciferase activity.

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# 32

## Human Hepatocyte Culture

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**Summary**

Primary culture of human hepatocytes is an in vitro model widely used to investigate numerous aspects of liver physiology and pathology. The technique used to isolate human hepatocytes is based on two-step collagenase perfusion. Originally performed *in situ* for obtaining hepatocytes from the adult rat, this technique has been adapted to the ex vivo treatment of human liver from organ donors or from lobectomy resection for medical purposes. This chapter describes experimental protocols for the isolation of hepatocytes from human liver tissue and for the preparation of short- and long-term cultures in which cells retain a differentiated phenotype for at least 1 mo. The various aspects emphasized here include the conditions for obtaining tissue, quality control of tissue for efficient perfusion, collagenase perfusion parameters, solutions for perfusion and culture media, cell substrate, cell plating, specific equipment, and safety conditions.

**Key Words:** Hepatocytes; collagenase; perfusion; primary culture; long-term culture; differentiation.

### 1. Introduction

Primary culture of hepatocytes is an in vitro model widely used to investigate various aspects of liver physiology and pathology ***(1)***. In particular, such cultures have been extensively used for assessing the expression and function of drug-metabolizing enzymes including cytochromes P450 (CYP), drug metabolism, drug–drug interactions, and the mechanisms of cytotoxicity and genotoxicity. Most of these studies have been carried out with rodent hepatocytes. However, because of species specificity in both the regulation and activity of drug-metabolizing enzymes, extrapolation from animals to humans is not generally possible. For this reason, several groups have developed human hepatocyte culture systems ***(2–8)***. The technique used to isolate human hepatocytes is based on the two-step collagenase perfusion first introduced by Berry and Friend ***(9)*** and modified by Seglen ***(10)***. Originally performed *in situ* for

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obtaining hepatocytes from the adult rat, this technique of perfusion has been adapted to the ex vivo treatment of human liver tissue. The aim of this chapter is to describe our experience in the isolation of hepatocytes from human liver tissue and the preparation of short- and long-term cultures.

### 1.1. Human Liver Samples

The use of human liver samples for hepatocyte preparation for scientific purposes must be approved by national ethics committees or other regulatory authorities.

Because of the extensive use of donor livers for transplantation, the availability of whole human liver has dramatically decreased in the last few years. Some donor livers are considered by surgeons to be unsuitable for transplantation (owing to, e.g., a high level of steatosis or cholestasis); however, these are generally not suitable for hepatocyte isolation either (*see* **Note 1**).

Our main source of human liver tissue therefore consists of lobectomies or smaller fragments resected for medical purposes. In general, the pathologies requiring such resections include primary tumor, metastasis, adenoma, angioma, or hydatid cyst. This type of sample has several advantages: first, because it would otherwise be discarded, the impact of ethical considerations is greatly reduced. Second, it is generally used within 4 h of removal (and in many occasions within 1 h), the cold ischemia period thus being reduced in comparison with that for whole donor livers. Third, anatomopathological data are available. Fourth, a sufficient amount of histologically normal tissue is generally available.

### 1.2. Requirements

The quality of the perfusion is critical for hepatocyte isolation. To this end, several requirements concerning the liver sample itself must be fulfilled. First, minimum leakage (if any) must occur during perfusion. For this purpose, the sample must be encapsulated in Glisson's capsula on all areas except, obviously, the edge left by the surgeon. In the case of lobectomy resection, the part of the tissue encompassing the tumor(s) is dissected by the surgeon in the operating theater and sent to anatomopathologists, whereas the remaining downstream encapsulated part is used for hepatocyte preparation (*see* **Note 2**). Second, several veins must be apparent on the cut edge; these will be used for perfusion. In some cases, these will have been sutured by the surgeon during the operation, to avoid excessive bleeding, and must be reopened for perfusion (*see* **Note 3**). Third, the mass of the liver sample is critical. It should be between approx 50 and 400 g. The problem with small samples is the difficulty of finding good vascular entries to obtain satisfactory perfusion. The problem with samples that exceed 400 g is that the ratio of the mass of tissue to the mass of collagenase is too high. This results in a decrease in the yield and quality of hepatocytes. When a whole liver is used, it is better to split it into two or three smaller pieces each meeting the previous two requirements. Before beginning the perfusion, fresh tissue samples are collected, stored in liquid nitrogen, and used for comparisons in further studies. Fourth, when the tissue is collected, it must be placed in a sterile plastic bag containing a sufficient volume of saline solution to overlay it and then be transported on ice.

## 2. Materials

### *2.1. Buffers and Solutions*

Buffers and solutions are prepared in deionized water, sterilized by passing through 0.22-µm filters, and stored in refrigerated stoppered bottles. All reagents are purchased from Sigma except penicillin/streptomycin, fungizone, and phosphate-buffered saline (PBS) (Gibco Invitrogen); selenium acetate (Aldrich); glucagene (Novo Nordisk); epidermal growth factor (EGF) (Peprotech); and Phagosurf DD (Phagogene DEC).

1. HEPES buffer: 10 m*M* HEPES, 136 m*M* NaCl, 5 m*M* KCl, 0.5% (w/v) glucose, pH 7.6.
2. EGTA solution: 0.5 m*M* EGTA in HEPES buffer (*see* **item 1**).
3. Antibiotic solution: penicillin (10,000 U/mL), streptomycin (10 mg/mL). Add 10 mL/L to HEPES buffer and to the EGTA solution.
4. Fungizone solution: fungizone (250 µg/mL). Add 3 mL/L to HEPES buffer and to the EGTA solution.
5. 70 m*M* $CaCl_2$ solution: add to HEPES buffer for collagenase solution.
6. BSA-HEPES solution: dissolve 5 g of bovine serum albumin (BSA) (fraction V)/L of HEPES buffer. Supplement with antibiotics and fungizone.
7. Phagosurf DD (Phagogene DEC), 0.25% solution in water: this product inactivates viruses including hepatitis B virus (HBV) and human immunodeficiency virus (HIV), as well as other pathogens, in <12 h.

### *2.2. Culture Media*

We use two different chemically and hormonally defined culture media for short- (approx 1 wk) and long-term (approx 1 mo) cultures. These media, first described by Isom and Georgoff ***(11)*** and Lanford et al. ***(12)*** for the culture of rat and monkey hepatocytes, respectively, have been adapted for human hepatocyte cultures in our laboratory ***(13,14)***. The short-term culture medium is used for preparation and plating of hepatocytes and for studies requiring only a few days (<7), such as CYP induction in response to xenobiotics. Other investigations requiring longer culture times are carried out in the long-term culture medium. Note that the long-term culture medium is three times more expensive than the short-term culture medium.

#### *2.2.1. Short-Term Culture Medium*

1. Ham's-F12 medium: dissolve the amount of powdered medium required for 5 L in approx 4.5 L of deionized water. Add 5.88 g of $NaHCO_3$, and after 15 min of bubbling with a mixture of 95% $O_2$ and 5% $CO_2$, adjust the pH to 7.4. Adjust the volume to 5 L with deionized water.
2. Williams' medium E: dissolve the amount of powdered medium required for 5 L in approx 4.5 L of deionized water. Add 11 g of $NaHCO_3$, and after 15 min of bubbling with a mixture of 95% $O_2$ and 5% $CO_2$, adjust the pH to 7.4. Adjust the volume to 5 L with deionized water.
3. Combine Ham's-F12 and Williams' E media and sterilize by passing through a 0.22-µm filter. Keep the mixture refrigerated in the dark in 1-L stoppered bottles. Ham's-F12 and Williams' E media may also be purchased (but at a higher price) as liquid media.
4. Pepare the mix of additives (*see* **Subheading 2.2.2.**) for complementing 25 L of Ham's-F12/Williams' E medium by combining submixes, 1, 2, and 3 and 75 mL of sterile

fungizone solution (total volume: 925 mL) (*see* **Note 4**). Aliquot the mix in 37-mL fractions and store at –80°C.

5. Prepare the final chemically and hormonally defined short-term culture medium: just before use by supplementing 1 L of Ham's-F12/Williams' E medium with 37 mL of mix and 2 mL of vitamin C solution.

### 2.2.2. Submixes of Additives for Short-Term Culture Medium

1. Submix 1: Dissolve 8.75 g of glutamine, 31.5 g of glucose, $2.5 \times 10^6$ U of penicillin, and 2.5 g of streptomycin in 500 mL of water, and sterilize the solution by passing through a 0.22-µm filter.
2. Submix 2: Dissolve 1.1 g of sodium pyruvate, 1 mg of dexamethasone (dissolved in 500 µL of ethanol), and 1.25 g of transferrin in a final volume of 75 mL of water.
3. Submix 3: Dissolve 100 µL of ethanolamine, 50 mg of insulin (dissolved in 10 mL of water containing 100 µL of glacial acetic acid), 5 mg of glucagon (dissolved in 10 mL of water containing 100 µL of 1 *M* NaOH), and 37.5 mg of linoleic acid in a final volume of 25 mL of water.
4. Vitamin C solution: 50 mg in 2 mL of water. Sterilize by passing through a 0.22-µm filter. Prepare just before use.

### 2.2.3. Long-Term Culture Medium

1. Dissolve one 5-L dose of powdered of Williams' E medium in approx 4 L of deionized water. Add 11.9 g of HEPES and 11 g of $NaHCO_3$, and adjust the pH to7.2. Adjust the volume to 5 L with deionized water.
2. Sterilize by passing through a 0.22-µm filter and keep refrigerated in the dark in 500 mL stoppered bottles.
3. Prepare the mix of additives (*see* **Subheading 2.2.4.**) for complementing 5 L of Williams' E medium by adding in a final volume of 250 mL of Williams' E medium: 10 mL of BSA-linoleic-linolenic solution, 5 mL of insulin solution (50 mg), 1 mL of transferrin solution (25 mg), 50 µL of selenium acetate solution (64 µg), 200 µL of dexamethasone solution (0.2 mg), 100 µL of liver growth factor solution (100 µg), 250 µL of cyclic adenosine monophosphate (cAMP) solution (12.25 mg), 500 µL of prolactin solution (PRL) (50 IU), 100 µL of ethanolamine solution (0.3 µg), 5 mL of glucagon solution (5 mg), 250 µL of EGF solution (250 µg), 50 mL of glutamine solution (1.46 g), and 50 mL of penicillin/streptomycin solution (500,000 U and 500 mg, respectively) (*see* **Subheading 2.1., item 3**). Sterilize the mix by passing through a 0.22-µm filter (PES; Nalgene). Add 15 mL of sterile fungizone (*see* **Subheading 2.1., item 4**). Aliquot the mix in 25-mL fractions and store at –80°C (*see* **item 3**).
4. Prepare the final chemically and hormonally defined long-term culture medium is prepared just before use by supplementing 500 mL of Williams' E medium with 25 mL of mix.

### 2.2.4. Additives for Preparing Mix for Long-Term Culture Medium

1. BSA-linoleic-linolenic acid solution: dissolve 1 g of BSA (fraction V) in a final volume of 10 mL of PBS. Add 20 µL of linoleic acid and 20 µL of linolenic acid.
2. Insulin: 100 mg in 10 mL of 1% (v/v) acetic acid.
3. Transferrin: 100 mg in 4 mL of water.
4. Selenium acetate: 6.45 mg in 5 mL of water.
5. Dexamethasone: 1 mg in 1 mL of dimethyl sulfoxide (DMSO).

6. Liver growth factor: 500 µg in 500 µL of Williams' E medium.
7. cAMP (N6,2'-*O* Dibutyryladenosine 3'-5' cyclic monophosphate): 49 mg in 1 mL of water.
8. PRL (luteotropic hormone): 250 IU in 2.5 mL 10 m*M* chlorhydric acid.
9. Ethanolamine: 3 µL in 1 mL of DMSO.
10. Glucagon: 5 mg in 5 mL of water.
11. EGF: 500 µg in 500 µL of water.
12. Glutamine solution: 200 m*M* (from Sigma).

### 2.3. Collagenase Solution

1. Prepare 1 L of HEPES buffer supplemented with antibiotics, fungizone, and 10 mL of 70 m*M* $CaCl_2$, and divide into two parts of 250 and 750 mL.
2. Dissolve 500 mg of collagenase in the 250-mL aliquot of this buffer, and sterilize by passing through 0.45- and 0.22-µm filters if necessary (*see* **Note 5**). Because of the cost of collagenase, this solution should be prepared only when perfusion of the tissue has been shown to proceed correctly (*see* **Subheading 3.2., step 4**).
3. Add this mixture to the 750-mL aliquot of HEPES buffer. This solution of collagenase will be used to dissociate the liver tissue.

### 2.4. Cell Culture Materials

1. Type I collagen–coated dishes (60 or 100 mm in diameter, or 6-, 12-, or 24-well plates) from Beckton Dickinson.
2. Fetal calf serum (FCS) tested for hepatocyte cultures.
3. Laminar-flow microbiology safety cabinet.
4. Nylon filter (250 mesh) sterilized by autoclaving.
5. Perfusion vessel (Pyrex or stainless steel), rubber tubing (hoses), Teflon terminal tip, and stoppers that can be sterilized by autoclaving.
6. Thermostated water bath for buffers and solutions, heater for perfusion vessel.
7. Pump for tissue perfusion with flux adjustment between 10 and 500 mL/min.
8. Vacuum liquid-aspiration device (for removal of liquid waste).
9. Waste collectors for tissue, liquids (blood, perfusion effluents), and other solid materials (undigested tissue, gloves, Whatman paper, aluminum foil, and so on).
10. Decontamination reservoir (50 L) for dissection instruments, perfusion vessel, tubing, and other reusable materials.
11. Standard apparatus for cell culture: incubators, low-speed centrifuge, optical microscope, rotary agitator, and so on.

## 3. Methods

### 3.1. Safety Conditions

Virological analysis of the patient from whom the liver sample has been resected must be carried out shortly before or at the time of the operation. The serologies include hepatitis A virus, HBV, and hepatitis C virus (HCV), and HIV. All laboratory staff should be vaccinated against HBV and clearly informed of the possible risks of infection.

Even when the virological analysis is negative, all experimentation with human tissue samples must conform to the safety policies regarding the protection of staff and the containment standard of the equipment and of the laboratory rooms in which tissue processing and isolation of and experimentation on cell cultures are to be performed (European standard containment laboratory type L2). In cases in which donor tissue is

infected with a hepatotrophic virus, isolation and culture must be performed in a containment laboratory type L3 (*see* **Note 6**).

All steps of isolation and culture of hepatocytes are carried out in a laminar vertical-flow microbiology safety hood, to protect not only the staff from contamination, but also the liver sample. Staff must wear sterile gloves, glasses, masks, and disposable coats and boots. All materials and liquid wastes must be decontaminated before discarding or resterilizing by autoclaving (for recycled materials). Instruments and materials to be reused are decontaminated by immersion in Phagosurf DD (0.25% solution) (final concentration) for 24 h. Prepare 50 L of this solution in an appropriate reservoir shortly before isolation of hepatocytes. Liquid wastes are stored in an appropriate reservoir in the presence of Phagosurf DD (0.25% solution) for 24 h. Other materials, such as used culture dishes, are decontamined by autoclaving before discarding.

### 3.2. Perfusions

On arrival in the laboratory, the liver sample is placed in a perfusion vessel and the edge is carefully examined in order to locate the various vein and artery sections that will be used for perfusion (*see* **Note 3**). The volumes indicated in the following protocol for buffers and solutions are adequate for a sample of approx 300 g; for smaller or larger samples, these should be modified accordingly. All solutions and buffers are kept at 37°C except the albumin-HEPES solution used for hepatocyte washings. Do not oxygenate solutions and buffers before perfusion, because this will generate oxidative stress in the tissue.

1. Wash the tissue with 1 to 2 L of HEPES buffer supplemented with antibiotics and fungizone (*see* **Subheading 2.1., item 1**) at a rate of approx 1 mL/min·g of tissue with no recirculation. In this step, blood is washed away (if this has not previously been carried out with saline buffer in the operating theater), the tissue is warmed to 37°C, and it can be ensured that perfusion is proceeding correctly (*see* **Note 7**). During this and further perfusion steps, the cannula is inserted successively in all veins/arteries present on the edge for approx 30 s each (one vein/artery at a time). Care must be taken not to damage the vein/artery section during this operation.
2. If perfusion is proceeding normally, start preparation of collagenase solution (*see* **Subheading 2.3.**).
3. Perfuse the tissue with 1 L of EGTA solution (*see* **Subheading 2.1., item 2**), supplemented under the same conditions as in **step 1**, with no recirculation.
4. Perfuse the tissue with 1 L of supplemented HEPES buffer (*see* **Subheading 2.1., item 1**), to remove the EGTA, under the same conditions as in **step 1**. At the end of this step, empty the reservoir of the perfusion vessel and wash several times with water.
5. Perfuse the tissue with the collagenase solution under the same conditions as in **step 1** except recirculate the solution and reduce the rate of perfusion to 100 mL/min. This step lasts a maximum of 20 min. During this step, softening of the tissue gradually appears as well as marbling, indicating that dissociation is proceeding efficiently (*see* **Note 8**).

### 3.3. Isolation and Washing of Hepatocytes

1. At the end of the collagenase perfusion, transfer the liver sample into a new stainless steel vessel, and open the Glisson's capsula in several places. Remove tumor(s) or metastases, if present, and send to an anatomopathologist (*see* **Note 2**).

2. Gently disrupt the tissue with scissors.
3. Complement the homogenate with 1 to 2 L of BSA-HEPES solution (see **Subheading 2.1., item 6**).
4. Filter the homogenate through a nylon filter (250 mesh), and distribute the filtrate into 150-mL centrifuge tubes. Wash the filter twice with approx 200 mL of BSA-HEPES solution to collect the hepatocytes that are trapped in the undissociated tissue homogenate.
5. Centrifuge the tubes for 5 min at 50*g* at room temperature.
6. Discard tyhe supernatant and gently resuspend the pellet, representing the hepatocytes, in 200 mL of BSA-HEPES solution per tube by five successive up and down runs with a pipet (*see* **Note 9**).
7. Repeat **steps 5** and **6**, are twice. At the end of the last centrifugation, the yield of the preparation may be roughly estimated by measuring the volume of the pellet: 1 mL of pellet represents approx $10^8$ cells. For more precise counting of cells, see **steps 9** and **10**.
8. At the end of the last washing, resuspend the pellet in an equal volume of BSA-HEPES solution and homogenize gently with a pipet as described in **step 6**.
9. Disperse 500 µL of hepatocyte suspension in 9.5 mL of the short-term culture medium. Place 250 µL of this suspension in a polystyrene tube and supplement with 50 µL of a 1% Trypan blue solution. After 2 min at room temperature, placed a 10-µL aliquot of this suspension in the compartment of a hemocytometer cell for counting.
10. Evaluate the yield and viability of the cells by examination under a microscope using a Trypan blue exclusion test. In our hands, the yield and viability are, on average, $7 \times 10^6$ cells/g of liver tissue and 85%, respectively (*see* **Note 10**).

### 3.4. Plating and Culture of Hepatocytes

1. After evaluation of yield and viability, complement an appropriate volume of short-term culture medium with FCS (5% in volume).
2. Dilute the hepatocyte suspension in this medium to 3.5 or $10 \times 10^6$ viable cells/mL for plating in 60- or 100-mm-diameter culture dishes, respectively.
3. Distribute the culture dishes on stainless steel trays (7 dishes of 100 mm or 18 dishes of 60 mm per tray), and add 2 or 7 mL of culture medium per 60- or 100-mm dish, respectively (use appropriate volumes for smaller wells).
4. Add 1 mL of an appropriately diluted suspension of cells (3.5 or $10 \times 10^6$ viable cells) per dish (use adequate sizing for smaller wells). This number of cells per dish corresponds approximately to a cell density of $12.5 \times 10^4$ cells/cm$^2$ for a confluent monolayer (*see* **Note 11**). Care must be taken to rehomogenize the cell suspension frequently by gentle circular agitation while distributing to the culture dish.
5. Evenly distribute the cells on the dish by gentle agitation (*see* **Note 11**).
6. Place the culture dishes in an incubator in a humid atmosphere of air 5% $CO_2$ at 37°C.
7. After 4 h, discard the serum-supplemented medium and replace with 3 or 8 mL of new serum-free medium per 60- or 100-mm dish, respectively (*see* **Note 12**).
8. Renew the culture medium every 24 h for short-term cultures and every 48–72 h for long-term cultures (*see* **Note 13**).

## 4. Notes

1. With high levels of steatosis or cholestasis, we generally found either poor yield, poor viability (<65%), and poor attachment of cells to culture dishes, or no dissociation at all.
2. This requires good coordination with the surgeon and the anatomopathologist. In all cases, an identification number is ascribed to the liver tissue so that the anonymity of the patients

(or donors) is fully respected. Only information on the gender, age, medical reasons for the surgical operation (liver lobectomies), or cause of death (liver donors) is communicated by the hospital. The subjects cannot be identified, directly or through identifiere important, pathological examination of the surgical specimen must in no way be hindered by the procedure used to isolate hepatocytes.

3. The most convenient manner of proceeding here is to make a new edge (by cutting a 0.5- to 1-cm-thick slice) parallel to the one left by the surgeon. This reveals various vascular entries.
4. Because of the numerous additives required to supplement the basal culture medium, it is preferable to prepare a concentrated solution of all the additives in either one or several submixes.
5. We only use batches of collagenase with a specific activity >400 U/mg. Some batches appear to be contaminated by microorganisms. This is assessed by microscopic examination of an aliquot of culture medium supplemented with 1 mg of collagenase after 96 h of incubation at 37°C under normal culture conditions. In such cases, the collagenase solution must be sterilized just before use (*see* **Subheading 3.2.**) either directly by passing through a 0.22-µm filter or, to prevent the filter from becoming clogged, first through a 0.45-µm filter and then through a 0.22-µm filter.
6. We have prepared and cultured hepatocytes from a patient who was infected with HCV. Yield, viability, and culture aspect were not significantly different from those observed with healthy liver.
7. This is checked by touching the tissue on perfusion and verifying the homogeneous warming of the liver sample. Should a part of the sample remain cold during this step, this means that the vein irrigating this area either has not been found (and it would be necessary to search for it to improve the yield of preparation) or is obstructed by a clot. In the latter case, a reperfusion at a higher rate may be tried (care must be taken not to damage the tissue by excessive pressure).
8. It is important to monitor this step by direct touching of the tissue. Correct perfusion and dissociation is revealed by swelling of the various areas of the sample when moving from one vein to another.
9. The pellet is generally formed by one "tight" part in the bottom and one "soft" part above. The soft part contains damaged cells and erythrocytes, which should be removed. This is performed by slowly tilting the tube. The soft pellet may represent up to 30% of material at the first washing step. Its volume gradually decreases thereafter to become negligible at the third washing. Removal of the soft pellet is critical for obtaining a preparation with a viability >80%, and a high purity.
10. As a general rule, we have observed that the quicker the procedure for the isolation of hepatocytes, the better the quality of cells and subsequent cultures. Therefore, isolation must be performed as rapidly as possible. Indeed, here, time is quality. Nevertheless, the yield and viability are widely variable from one sample to another and range between 1 and $10 \times 10^9$ cells, and 70 and 90%, respectively. The reasons for this variability are difficult to identify; parameters such as duration of cold ischemia, health status of the liver, pathology, formation of intratissue clots, or quality of perfusion (number and state of vessels used for perfusing) could possibly be involved.
11. The differentiated hepatic phenotype is maintained in confluent but not in subconfluent cultures ***(15)***. Establishment of a homogeneous confluent-cell monolayer throughout the dish is therefore critical. Once the dishes present on one tray are supplemented with culture medium and cells, the tray is held horizontally and gently agitated from left to right (1 cycle/s for 10 s) and back and forth (1 cycle/s for 10 s) with a pause of 5 s in between.

This allows the suspension to be spread homogeneously on the dish and avoids rotational movement of the suspension, which would result in preferential distribution of cells toward the periphery of the dish with low density in the center.

12. The culture medium must be aspirated and poured gently to avoid detachment of cells. Changes in the medium will be facilitated by placing the trays on an inclined plane.
13. Regarding phenotype characterization, several markers of hepatic phenotype have been investigated and shown to be maintained in our cultures for at least 1 wk (short-term cultures) and 5 wk (long-term cultures). These include the following:
    a. The induction of CYP genes by prototypic inducers and expression of nuclear receptors involved in the control of induction (short- and long-term cultures) ***(13,14,16–24)***.
    b. The oxidative metabolism of drugs and reconstitution of drug–drug interactions (short- and long-term cultures) ***(25–27)***.
    c. The production of albumin (short- and long-term cultures), and α1-antitrypsin, fibrinogen, and plasminogen (long-term culture) ***(14).***
    d. The production of apolipoproteins ApoA, ApoB, lpa (short- and long-term cultures) ***(14)***.
    e. The production of acute-phase proteins (ceruloplasmin, fibrinogen, ferritin) in response to cytokines interleukin (IL) –1, IL–6, and tumor necrosis factor (short-term culture) ***(28)***.
    f. The production of blood coagulation factors (long-term culture) ***(29)***.
    g. The expression of C/EBP factors (long-term culture) ***(30)***.
    h. The mitotic response of cells to specific hepatotrophic factors including EGF, transforming growth factor α, hepatocyte growth factor, and serum from patients with liver failure (short-term culture, under low cell density) ***(31–33)***.
    i. In vitro infection by HCV and hepatitis D virus (long-term culture) ***(34–36)***.

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# 33

# Determination of Cellular Localization of Expression of Flavin-Containing Monooxygenase Genes in Mouse Tissues by *In Situ* Hybridization

**Azara Janmohamed, Ian R. Phillips, and Elizabeth A. Shephard**

**Summary**

Methods are described for the cellular localization of expression of flavin-containing monooxygenase (FMO) genes in various mouse tissues by *in situ* hybridization. These include the production of digoxigenin (DIG)-labeled antisense and sense RNA probes by transcription from FMO cDNA templates, the preparation of paraffin wax-embedded and cryostat tissue sections, the hybridization of RNA probes to tissue sections, and the specific detection of hybridized probes using an antibody to DIG.

**Key Words:** Flavin-containing monooxygenase; antisense RNA probes; *in situ* hybridization; mouse; digoxigenin-labeled probes; gene expression.

## 1. Introduction

Differences in the patterns of expression of drug-metabolizing enzymes such as the cytochromes P450 (CYPs) and flavin-containing monooxygenases (FMOs) have implications for the ability of organisms to respond to substrates of these enzymes that are present in their environment and diet, or that are used as therapeutic drugs in clinical or veterinary medicine. Members of the FMO family exhibit differential developmental stage- and tissue-specific patterns of expression, which differ among species ***(1–9)***. In addition, the expression of FMOs is controlled in a cell-type-specific manner ***(9–13)***. In mouse liver, the distribution of FMO1 and FMO5 ***(9)*** is similar to that of other phase I enzymes, such as the CYPs, most of which are more highly expressed in hepatocytes of the perivenous region ***(14)***. By contrast, FMO2, FMO4, and FMO3 mRNAs are localized to the periportal region ***(9)***. The localization of expression of FMO3 to the periportal region of the liver is unusual for a protein whose role is considered to be predominantly one of xenobiotic metabolism. The location of FMO3 within the liver may contribute to increased toxicity of potentially harmful chemicals

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activated by FMO3. For example, thiourea, phenylthiourea, and α-naphthylthiourea are toxic to mouse C3H/10$T_{1/2}$ cells expressing human FMO3, but not to those expressing human FMO1 ***(15)***. The antifungal agent ketoconazole is reported to cause hepatotoxicity in humans and rabbits and is a substrate for several FMOs ***(16,17)***. Such observations indicate that the differential pattern of FMO expression (and of other drug-metabolizing enzymes) within the liver lobule should be considered when carrying out both in vivo and in vitro xenobiotic metabolism studies on substances that are substrates for these enzymes.

In this chapter, we describe the use of nonradioactive, digoxigenin-labeled antisense probes to determine the cellular localization of FMO mRNAs in various mouse tissues including the liver, lung, and brain. For an excellent review on the theory and practice of *in situ* hybridization (ISH), *see* **ref.** ***18***.

## 2. Materials

### 2.1. Preparation of Riboprobe Template

1. pBluescript (Stratagene Europe, Amsterdam, The Netherlands) plasmid containing the appropriate cDNA fragment (*see* **Note 1**).
2. Restriction endonucleases, for linearization of plasmids.
3. 7.5 *M* Ammonium acetate (filter sterilized).
4. Buffered phenol (pH 6.0–8.0) (Fisher Scientific UK, Loughborough, UK):chloroform (1:1 [v/v]) (*see* **Note 2**).
5. Isopropanol.
6. 70% (v/v) Ethanol.

### 2.2. In Vitro Transcription of Riboprobe From Template

1. DIG RNA labeling kit (SP6/T7) (Roche, Lewes, UK): this kit comes with all the reagents required to carry out the in vitro transcription reaction, as well as a control template DNA encoding the *Neo* gene.
2. T3 RNA polymerase (Stratagene Europe).
3. 0.5 *M* Ethylenediamine tetra-acetic acid (EDTA).
4. Fresh 2% (w/v) agarose gel: prepare the gel using equipment that is clean and relatively RNase free.

### 2.3. Processing and Sectioning of Tissue

#### 2.3.1. Paraffin Wax-Embedded Sections

1. Phosphate-buffered saline (PBS): prepare a 1X solution by dissolving PBS tablets (Sigma-Aldrich, Poole, UK) in distilled water per the manufacturer's instructions.
2. Diethylpyrocarbonate (DEPC)-PBS: add 1 mL of DEPC/L of PBS, incubate at 37°C overnight, and autoclave.
3. Formaldehyde, 37% (v/v) solution (Formalin) (Sigma-Aldrich).
4. 10% (v/v) Formalin: prepare by mixing 1 part 37% formalin with 9 parts DEPC-PBS.
5. DEPC-treated water: add 1mL of DEPC/L of distilled water, incubate at 37°C overnight, and autoclave.
6. 30, 50, 70, 90, and 100% (v/v) ethanol (all made up in DEPC-treated water).
7. Histo-Clear™ II (Flowgen, Ashby de-la Zouch, UK).
8. Histo-Clear II:ethanol (1:1 [v/v]).

9. Paraplast embedding medium (wax) (Sigma-Aldrich): melt solid wax pellets, in an appropriate container, in a 60°C embedding oven or water bath.
10. Wax:Histo-Clear II (1:1 [v/v]): dilute 1 part melted wax to 1 part Histo-Clear II.
11. Wax:Histo-Clear II (3:1 [v/v]): dilute 3 parts melted wax to 1 part Histo-Clear II.
12. Embedding oven set to 60°C.
13. Hot plate set to 60°C.
14. Plastic molds to embed tissues.
15. Microtome.
16. Superfrost Plus Microscope slides (VWR, Lutterworth, UK).

#### *2.3.2. Cryosections*

1. DEPC-PBS.
2. 10% (v/v) Formalin in DEPC-PBS.
3. Tissue-Tek® OCT Compound (Agar Scientific, Stansted, UK).
4. Cryostat.
5. Superfrost Plus Microscope slides (VWR).

### *2.4. Preparation of Tissue Sections For Hybridization*

1. Glass Coplin jars.
2. 100, 75, 50, and 25% (v/v) Ethanol: dilute ethanol in DEPC-PBS (*see* **Subheading 2.3.1., item 2**).
3. 4% (w/v) Paraformaldehyde (Sigma-Aldrich) in DEPC-PBS: in a fume hood, dissolve the appropriate amount of paraformaldehyde in DEPC-PBS. Heat gently and add a couple of pellets of NaOH. Paraformaldehyde is soluble only at an alkaline pH. Once dissolved, cool the solution and filter through No. 1 Whatman filter paper.
4. Proteinase K (Roche): this is supplied as a solution, the concentration of which may vary from batch to batch.
5. Xylene (VWR).

### *2.5. Hybridization With DIG-Labeled Probes*

1. 10X "salts": 2 *M* NaCl, 100 m*M* Tris-HCl, pH 7.5, 50 m*M* $NaH_2PO_4$, and 50 m*M* $Na_2HPO_4$.
2. Baker's yeast tRNA (Roche): it is important to use high-quality tRNA that dissolves easily to produce a colorless rather than yellow solution.
3. 50% (w/v) Dextran sulfate (Sigma-Aldrich): dissolve the appropriate amount of dextran sulfate in DEPC-treated water by heating to 60°C in a water bath. The stock solution should be kept frozen at –20°C.
4. 50X Denhardt's solution (Sigma-Aldrich).
5. Hybridization buffer (*see* **Note 3**): 1X "salts," 50% (v/v) deionized formamide (Sigma-Aldrich), baker's yeast tRNA (0.1 mg/mL), 10% (w/v) dextran sulfate, and 1X Denhardt's solution. The volume should be made up with DEPC-treated water.
6. Cover slips: these must be baked or fresh, to ensure that there is no RNase contamination.
7. Bioassay dishes (245 × 245 × 25 mm) (Sigma-Aldrich): these are convenient to clean and hold at least eight slides each.
8. 20X saline sodium citrate (SSC): 175.3 g NaCl and 88.2 g of sodium citrate made up to 1 L with deionized water. The final concentrations of the ingredients are 3 *M* NaCl, 0.3 *M* sodium citrate.
9. Whatman 3MM paper wetted in 1X SSC, 50% (v/v) formamide.

### 2.6. Posthybridization Washes

1. Wash buffer: 1X SSC, and 50% (v/v) formamide, 0.1% (v/v) Tween–20 (Sigma-Aldrich).
2. 65°C water bath.
3. 5X MAB: 500 m*M* maleic acid (Sigma-Aldrich), 750 m*M* NaCl.
4. MABT: 1X MAB solution containing 0.5% (v/v) Tween-20.

### 2.7. Blocking and Incubation With Antibody

1. Sheep serum (Chemicon Europe, Chandlers Ford, UK): heat inactivate at 55°C for 30 min.
2. Blocking solution: 1X MABT containing 2% (w/v) blocking reagent (Roche), 20% (v/v) inactivated sheep serum. Dissolve the blocking reagent and MABT by heating to 65°C, and then add the appropriate volume of sheep serum. Once the solution is made it can be stored in aliquots at –20°C.
3. Anti-DIG alkaline phosphatase (AP)-conjugated antibody (Fab fragments) (Roche).
4. Antibody solution: anti-DIG AP-conjugated antibody (Fab fragments) diluted 1:1500 (v/v) in blocking solution.
5. DAKO PAP Pen (DakoCytomation, Ely, UK).

### 2.8. Postantibody Washes and Color Reaction

1. Prestaining buffer: 100 m*M* Tris-HCl, pH 9.0, 100 m*M* NaCl, and 5 m*M* $MgCl_2$. Prepare prestaining buffer without 5 m*M* $MgCl_2$ and store at room temperature. Add the $MgCl_2$ just before use.
2. 10% (w/v) Polyvinyl alcohol (PVA) (average mol wt = 70–100 kDa) (Sigma-Aldrich): dissolve the appropriate amount of PVA in prestaining buffer and incubate at 80°C for at least 6 h or overnight. Store at room temperature.
3. Staining buffer: Prestaining buffer, 5% (w/v) PVA, 0.2 m*M* 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche), and 0.2 m*M* nitroblue tetrazolium salt (NBT) (Roche). Prepare the staining solution just before use by adding equal volumes of a stock solution of 10% (w/v) PVA and prestaining buffer and then adding BCIP and NBT.

### 2.9. Poststaining Treatment

1. Graded series of ethanols (30, 60, 80, 95, and 100% [v/v]) made up in deionized water.
2. Xylene.
3. DPX mountant (VWR).
4. Cover slips.

## 3. Methods

The most important factor for success with *in situ* hybridization of RNA is that all the solutions, glass, and plasticware be RNase free. Solutions for tissue processing, prehybridization, and hybridization must be DEPC treated. All glassware and surgical instruments must be baked at 80°C before use. After hybridization these precautions need not be taken.

### 3.1. Preparation of Riboprobe Template

1. Digest approx 10 μg of the recombinant plasmid with an appropriate restriction enzyme that cuts the construct once 5' of the cDNA insert. It is important that the antisense probes generated be between 150 and 500 bp (*see* **Note 4**).
2. Check the digest by agarose gel electrophoresis, to ensure that >95% of the plasmid is linearized.

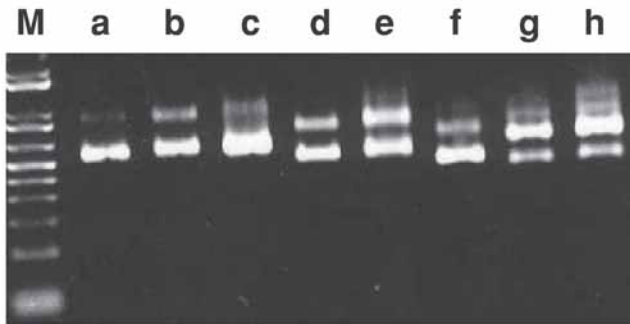


Fig. 1. Integrity of riboprobe. Mouse FMO riboprobes were analysed by electrophoresis through a 2% agarose gel. Lane M, 1-kb+ DNA marker; flanes a and b, FMO1 antisense and sense, respectively; lanes c and d, FMO3, antisense and sense, respectively; lanes e and f, FMO4 antisense and sense, respectively; and lanes g and h, FMO5 antisense and sense, respectively.

3. Add 0.5 vol of 7.5 *M* ammonium acetate and extract once with phenol/chloroform.
4. To the aqueous layer add an equal volume of isopropanol and allow the DNA to precipitate at room temperature for 5 min.
5. Centrifuge at 14,000*g* for 10 min at 4°C. Remove the supernatant and wash the DNA pellet in 70% (v/v) ethanol. Centrifuge as before. Pour off the ethanol. Leave samples to air-dry.
6. Resuspend the DNA in sterile, RNase-free water. Do not use DEPC-treated water, because DEPC interferes with the transcription reaction.
7. Measure the $A_{260}$ and determine the concentration of the linearized DNA.

### 3.2. In Vitro Transcription of Riboprobe From Template

1. Add the following components of the DIG RNA labeling kit to a 1.5-mL tube on ice: 1 µg of linearized template (*see* **Subheading 3.1.**), 2 µL of 10X NTP labeling mixture, 2 µL of 10X transcription buffer, 1 µL of RNase inhibitor, and 2 µL of RNA polymerase (SP6, T3, or T7) (20 U/µL) (*see* **Note 5**). Make up to 20 µL with sterile water. Mix the contents gently and incubate at 37°C for 2 h.
2. Add 2 µL of 0.5 *M* EDTA to stop the reaction. Make the volume of the reaction up to 100 µL with DEPC-treated water.
3. Electrophorese a 5-µL aliquot of the probe on a fresh 2% (w/v) agarose gel. Although the size of the transcript cannot be determined using such a gel, it provides a quick way of determining the integrity of the riboprobe (**Fig. 1**).

### 3.3. Processing and Sectioning of Tissue

All solutions, glassware, and surgical instruments must be RNase free. In our laboratory, we use paraffin wax-embedded sections, because retention of tissue morphology is much better in these than in cryosections. The only disadvantage of using paraffin-embedded tissues is the processing time required. The procedures detailed here for processing tissues have been optimized for ISH and give reproducible results without loss of signal and with no variability among different tissues.

### 3.3.1. Paraffin Embedding and Sectioning of Tissues

1. Anesthetize a mouse using standard techniques (*see* **Note 6**).
2. Perfuse the mouse (through the left ventricle of the heart) with fresh 10% formalin (prewarmed to 37°C).
3. Remove all the required tissues and fix in 10% formalin overnight.
4. The following day, wash the tissues twice for 20 min each time in DEPC-PBS.
5. Replace the PBS sequentially with 30, 50, and then 70% ethanol. Allow 30 min for each incubation (with shaking). If using the brain, increase incubation times to 1 h.
6. Remove the final ethanol wash and cover the tissue with fresh 70% ethanol. The tissues can then be stored in 70% ethanol at –20°C.
7. To continue the processing, remove the 70% ethanol and incubate the tissues in 90% ethanol for 30 min (allow 1 h for the brain). Wash twice in 100% ethanol for 15 min each time (increase incubation time to 30 min for the brain).
8. Transfer the tissues to Histo-Clear II:ethanol (1:1) for 10 min, followed by 100% Histo-Clear II for 5 min. Do not incubate the tissues for more than 5 min in Histo-Clear II, because this makes them brittle when sectioning is carried out.
9. Place the tissues in a 1:1 mixture of wax:Histo-Clear II at 60°C for 10 min.
10. Transfer to a 3:1 mixture of wax:Histo-Clear II at 60°C for 10 min and finally to wax (at 60°C) for about 30 min. Then place the tissues in fresh wax for a further 30 min. When working with brain tissue, increase the wax treatment to at least 3 to 4 h.
11. Using plastic molds placed on a hot plate, embed the tissues in fresh wax. Ensure that there are no bubbles and that the wax is liquid when embedding the tissues; otherwise, the tissue blocks will give uneven sections.
12. Using a microtome, cut 10-μm sections and remove creases in the ribbons of sections by floating on top of water in a water bath at 37°C. To place sections on slides, immerse Superfrost slides in the water bath (close to a ribbon of sections) and use the slides to lift the sections out. The sections will stick to the surface of the slides. Allow the slides to dry overnight at 37°C.

### 3.3.2. Cryosection Processing

1. Excise the tissue from the perfused animal (*see* **Subheading 3.3.1., item 2**).
2. Wash the tissue in DEPC-PBS; then fix in 10% formalin overnight.
3. Place OCT medium in a plastic mold and embed the tissues in the OCT medium. Place the mold inside a cryostat to solidify.
4. Mount the tissue block onto the cryostat and cut 10-μm sections.
5. Remove the creases in the ribbon of sections by gently teasing them out with a soft brush. Place a slide on the sections; they will attach immediately to the slide.
6. Air-dry the sections and store at –20°C.

## 3.4. Preparation of Tissue Sections For Hybridization

All procedures are carried out in baked, glass Coplin jars.

### 3.4.1. Paraffin Sections

1. To dewax the sections, place slides in xylene for 10 min. Repeat this step.
2. Transfer the slides to 100% ethanol for 3 min. Discard the ethanol. Repeat this step twice.
3. Hydrate the sections by incubating the slides sequentially in 75, 50, and then 25% ethanol for 3 min each time. Wash the slides twice in DEPC-PBS for 5 min each time.

4. Place the slides in 4% paraformaldehyde for 20 min. Wash three times in PBS for 5 min each time.
5. Dilute proteinase K to a final concentration of 10 µg/mL in PBS. A solution of 50 mL is sufficient to immerse slides in a Coplin jar (*see* **Note 7**). Incubate the slides for 10–15 min at 37°C.
6. Return the slides to 4% paraformaldehyde solution (the same solution as that used in **step 4** can be used) and incubate for 20 min (at room temperature).
7. Wash the slides three times for 5 min each time with DEPC-PBS. Dehydrate in the graded alcohol series used in **step 3** but in the reverse order (25, 50, 75, and then 100%) for 1 min each time.
8. Air-dry the slides. They are now ready for hybridization.

#### *3.4.2. Cryosections*

1. Before hybridization thaw the sections and allow them to come to room temperature.
2. Wash the sections three times in DEPC-PBS for 5 min each time. The sections are now ready for hybridization.

### *3.5. Hybridization With DIG-Labeled Probes*

1. Dilute DIG-labeled riboprobes in hybridization buffer. We usually use a dilution of about 1:1000 (v/v). Depending on the efficiency of the transcription reaction, the dilution may need to be varied; determine this empirically.
2. Denature the mixture of probe and hybridization buffer for 10 min at 70°C.
3. Vortex to mix and centrifuge briefly.
4. Place the slides flat (sections side upward) inside a Perspex box containing two sheets of Whatman 3MM paper wetted in 1X SSC, 50% formamide (*see* **Note 8**). Add 100–150 µL of probe to each slide. Gently lower cover slips onto the slides. Ensure that all the sections on the slides are covered with the hybridization mixture. Hybridize overnight at 55–65°C.

### *3.6. Posthybridization Washes*

1. Fill a Coplin jar with wash buffer and warm to 65°C.
2. Transfer the slides (do not remove the cover slips) to the Coplin jar, and incubate for 15 min or until the cover slips have fallen off, or slide off easily.
3. Wash the slides a further two times for 30 min each time in wash buffer at 65°C.
4. Wash the slides twice for 30 min each time in 1X MABT at room temperature.

### *3.7. Blocking and Incubation With Antibody*

1. Dry off the slides using tissue paper, being careful not to touch the sections.
2. Circle the sections using a DAKO PAP Pen.
3. Transfer the slides to a humidified chamber (*see* **Note 9**). Place the slides with the sections facing upward. Cover the sections with blocking solution (approx 300 µL/slide). Incubate the sections for at least 1 h at room temperature.
4. Remove the blocking solution by gently tipping it off the slides. Cover the sections with anti-DIG AP-conjugated antibody (Fab fragments) diluted 1:1500 (v/v) in blocking solution. Incubate the slides in this solution overnight at 4°C.

### *3.8. Postantibody Washes and Color Reaction*

1. Transfer the slides to Coplin jars containing MABT and wash five times for 30 min each time at room temperature.

2. Wash the slides twice for 10 min each time in prestaining buffer.
3. Incubate the slides in staining buffer at 37°C in the dark.
4. Check for staining every few hours until satisfactory. This can take from 3 to 48 h (*see* **Note 10**). Staining is observed as a purple color and can be clearly distinguished under a light microscope (**Fig. 2**).
5. To stop the reaction, remove the slides from the staining buffer and rinse several times in distilled water.

### 3.9. Poststaining Treatment

1. Dehydrate the sections sequentially through 30, 60, 80, 95, and then 100% ethanol for at least 1 min each.
2. Wash twice in xylene for at least 1 min each time. However, an overnight incubation in xylene gives the best results.
3. Place a cover slip on each slide using DPX mountant and leave for 24 h to set fully. Store the slides in the dark to prevent the color from fading.

## 4. Notes

1. Any plasmid containing promoters such as T3, T7, or SP6 can be used for the in vitro transcription reaction. However, we have experienced problems when carrying out in vitro transcription from cDNAs cloned into the TOPO-TA vector (Invitrogen, Paisley, UK).
2. The appropriate precautions should be taken when using phenol:chloroform. All extractions should be done in a fume hood and gloves should be worn.
3. This is the most crucial component of the ISH procedure and must be made with great care, ensuring no RNase contamination. The highest-quality reagents and water must be used.
4. Probes differ in their tissue permeability, depending on their length. We have observed problems with permeability of tissues when probes are longer than 500 bp. Sometimes probes derived from certain regions of a cDNA will not hybridize to a tissue. If staining is expected but not observed, try using a probe transcribed from a different region of the same cDNA.
5. When using T3 or T7 RNA polymerase from Stratagene, the enzyme must be diluted to 20 U/µL. If this is not done, the efficiency of transcription will be very low and the probe yield will decrease significantly.
6. All animal procedures must be carried out according to local regulations.
7. The concentration of proteinase K used may need to be varied from tissue to tissue. We have found that a concentration of 10 µg/mL works very well for all soft tissues, such as the liver and brain. Other tissues, such as muscle, may require higher concentrations of the enzyme. This must be determined empirically.
8. The mix of 1X SSC, 50% formamide, instead of water, is used to wet the Whatman paper, because this ensures that the atmosphere in which the slides are incubated is saturated with formamide vapors, which prevents the slides from drying out.
9. Any Tupperware or Perspex box can be used for this. The slides should not touch the bottom of the box. To achieve this, disposable pipets can be placed horizontally inside the box and the slides placed on top of the pipets.
10. The inclusion of 5% PVA in the staining buffer reduces background and intensifies the signal, even after a long period of incubation. In our experience, incubation at 37°C for 24 h is sufficient to detect rare mRNA molecules.

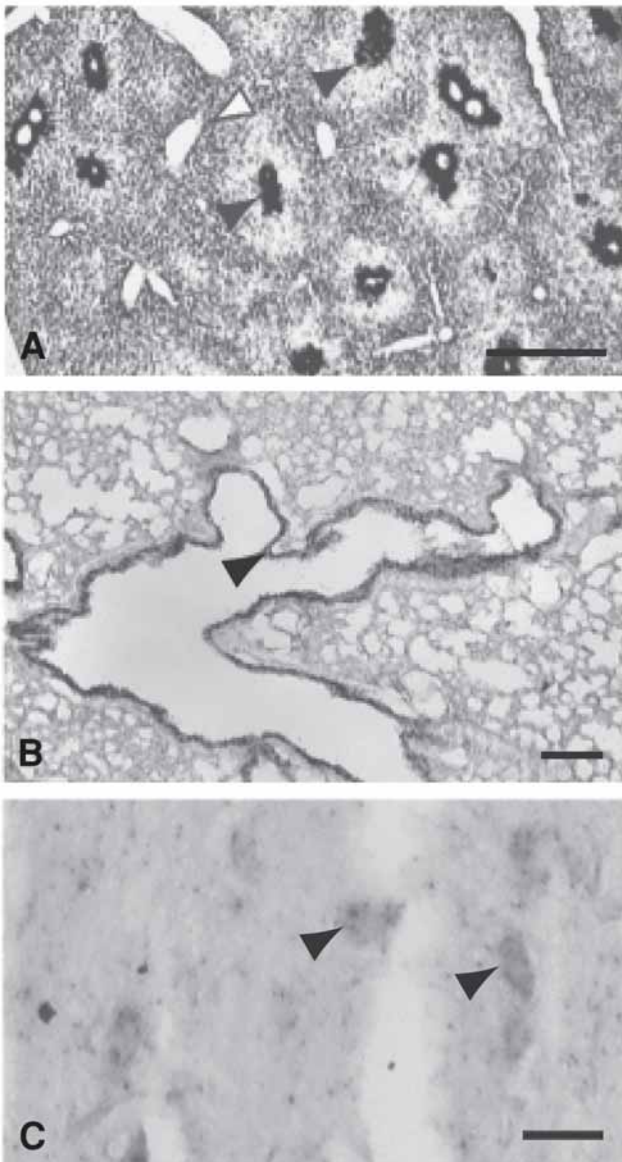


Fig. 2. Localization of FMO mRNAs in mouse liver, lung and brain. **(A)** Female mouse liver was hybridized with antisense probes for FMO3, and glutamine synthetase, which served as a control for perivenous expression. Black arrowheads indicate the central veins and the white arrowhead the portal vein. Bar = 200 µm. **(B)** Lung was hybridized with an antisense probe for FMO3 mRNA. The arrowhead indicates the terminal bronchiole. Staining is observed specifically in the epithelial cells lining the alveoli. Bar = 100 µm. **(C)** An antisense probe for FMO1 mRNA was hybridized with a section of the cerebrum of mouse brain. Arrowheads indicate specific staining observed in the neurons. Bar = 200 µm.

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# 34

# Deletion of Genes From the Mouse Genome Using Cre/*loxP* Technology

**Diana Hernandez, Pritpal Chandan, Azara Janmohamed, Ian R. Phillips, and Elizabeth A. Shephard**

**Summary**

The steps required to delete genes from the mouse genome are illustrated by showing how a cluster of three flavin-containing monooxygenase (*Fmo*) genes (*Fmo1*, *Fmo2,* and *Fmo4*) were deleted from mouse chromosome 1. Such large deletions are accomplished using *loxP*/Cre recombinase technology. Genomic clones corresponding to the genes to be deleted are first isolated, and then appropriate genomic fragments are cloned into vectors containing a *loxP* site. This produces targeting vectors, which are electroporated into mouse embryonic stem (ES) cells to allow a homologous recombination event to take place between the mouse genomic fragment, present within the vector, and the homologous sequences in the ES cell genome. Screening of ES cells for recombinants in which *loxP* sites have been inserted on either side of the gene cluster to be deleted is described. Recombination by Cre recombinase to produce ES cell lines carrying the deletion on chromosome 1 is also described.

**Key Words:** Chromosomal engineering; *loxP*; Cre recombinase; gene targeting; knockout mice; FMO; embryonic stem cells; flavin-containing monooxygenase; drug metabolism.

## 1. Introduction

Knockout (KO) mice that are deficient in genes for specific xenobiotic-metabolizing enzymes, provide useful experimental models to define the role of these enzymes in the metabolism of xenobiotics in an intact animal ***(1)***. In addition, comparison of gene expression patterns between wild-type (WT) and KO mice may provide insights into the role of xenobiotic-metabolizing enzymes in endogenous biochemical and physiological processes. We describe the use of the Cre/*loxP* system, which enables a gene, or a cluster of genes, to be deleted from a mouse chromosome. A gene(s) is deleted in mouse embryonic stem (ES) cells by first inserting, via homologous recombination, a *loxP* (locus of crossover of P1) ***(2)*** site on either side of the genomic segment to

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be deleted. Subsequent transfection of the ES cells with a plasmid expressing the protein Cre (causes recombination) recombinase ***(3)*** results in a site-specific recombination event between the two *loxP* sites and the loss of intervening (knocked-out) DNA. We describe the methods that we have used to delete a 107-kb region of mouse chromosome 1 that encompasses the genes encoding three flavin-containing monoooxygenases (FMO1, FMO2, and FMO4) ***(4)***. Homologous recombination is a rare event. Thus, the vectors described to target *loxP* sites to flank the *Fmo* genes also contain selectable markers (either neomycin or puromycin resistance) ***(5)*** to permit the isolation of ES cells that have been correctly targeted. In addition, a successful recombination event between the two *loxP* sites will reconstitute an *HPRT* minigene. Therefore, ES cells in which the correct gene rearrangement has occurred will survive on medium containing hypoxanthine, aminopterin, and thymidine (HAT). ES cells heterozygous for the required deletion are analyzed for karyotype as described in Chapter 35. Cells with a normal karyotype are injected into a 3.5-d-old blastocyst, which is then implanted into the uterus of a pseudopregnant female as described in Chapter 36.

## 2. Materials

### *2.1. Isolation and Characterization of Genomic Clones Containing* loxP *Sites*

#### *2.1.1. Screening of 5′ and 3′* hprt *Libraries*

1. 5' *hprt* mouse genomic DNA library ***(6)*** (*see* **Note 1**).
2. 3' *hprt* mouse genomic DNA library ***(6)*** (*see* **Note 1**).
3. NZCYM broth and agar capsules (Anachem, Luton, UK).
4. NZCYM broth supplemented with 0.2% maltose and 10 m*M* $MgSO_4$.
5. NZCYM bottom agar: 15% (w/v) agar in NZCYM broth.
6. NZCYM top agar: 7% (w/v) agar in NZCYM broth.
7. Falcon tubes (15 mL) (BD BioSciences Clontech, Oxford, UK).
8. Hybond-NX filters of the appropriate size for the Petri dishes used (Amersham Biosciences, Amersham, UK).
9. Denaturation solution: 58.4 g of NaCl and 20 g of NaOH in 1 L of deionized water.
10. Neutralization solution: 146 g of NaCl and 60.5 g of Tris base (adjust pH to 7.4) in 1 L of deionized water.
11. Stratalinker (Stratagene Europe, Amsterdam, The Netherlands).
12. Sodium phosphate solution: 71 g of $Na_2HPO_4$ and 60 g of $NaH_2PO_4$ in 500 mL of deionized water.
13. Church buffer: 250 mL of sodium phosphate solution, 35 g of sodium dodecylsulfate (SDS), 1 mL of 0.5 *M* ethylenediaminetetraacetate (EDTA), and 1% (w/v) bovine serum albumin (BSA) made up to 500 mL with deionized water.
14. 20X saline sodium citrate (SSC): 175.3 g of NaCl and 88.2 g of sodium citrate made up to 1 L with deionized water.
15. X-ray film: Hyperfilm MP (Amersham Biosciences).
16. SM buffer: 5.8 g of NaCl, 2 g of $MgSO_4.7H_2O$, 50 mL of 1 *M* Tris-HCl (pH 7.5), and 5 mL of 2% (w/v) gelatin in 1 L of deionized water.
17. *Escherichia coli* strains: XL 1-Blue MRA and XL 1-Blue MRA (P2) (Stratagene Europe).

#### 2.1.2. Circularization of Phage Clones Into Plasmids Using Cre-Expressing Bacteria

1. *E. coli* strain BNN132.
2. 1 *M* $MgCl_2$.
3. Luria-Bertani (LB) broth.
4. LB agar supplemented with ampicillin (50 µg/mL) and 0.1% (w/v) glucose.

#### 2.1.3. Analysis of Targeting Vectors

1. DNA purification kit.
2. Restriction endonucleases.
3. Buffered phenol: liquefied phenol washed with Tris buffer (Fisher, Loughborough, UK).
4. Chloroform.
5. Agarose gel electrophoresis equipment.
6. Hybridization probes.
7. Nylon membranes and solutions for hybridization of Southern blots.

### 2.2. Transfection of ES Cells With Targeting Constructs

1. ES cell line.
2. KO Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Paisley, UK) supplemented with 15% (v/v) fetal calf serum (FCS) (Foetal Calf Serum [European Origin]; TCS Biologicals, Buckingham, UK).
3. Gelatin-coated tissue culture plates (Sigma-Aldrich, Poole, UK).
4. 2-mercaptoethanol (Sigma-Aldrich).
5. Penicillin/streptomycin/glutamine (100X), liquid (Invitrogen).
6. Nonessential amino acids (100X), liquid (Invitrogen)
7. Leukemia inhibiting factor (LIF): ESGRO LIF (1000 U/mL) (Chemicon Europe, Chandlers Ford, UK).
8. Supplemented KO DMEM: KO DMEM supplemented with 15% (v/v) FCS, 0.1 m*M* 2-mercaptoethanol, penicillin (50 U/mL), streptomycin (50 mg/µL), 1 m*M* L-glutamine, and LIF (1000 U/mL).
9. DNA clones prepared for transfection (*see* **Note 2**).
10. Electroporator and cuvets (Bio-Rad, Hemel Hempstead, UK).
11. Trypsin solution: 1.25 g of porcine trypsin powder (Invitrogen), 0.2 g of EDTA, 3.5 g of NaCl, 0.595 g of $Na_2HPO_4$, 0.12 g of $KH_2PO_4$, 0.185 g of KCl, 0.5 g of D-glucose, 1.5 g of Tris, 0.5 mL of phenol red (Sigma-Aldrich) made up to 500 mL with tissue-culture-grade water. Adjust the pH to 7.6 with HCl. Filter sterilize using a 0.2-µm filter and store in aliquots at –20°C.
12. 1X Phosphate-buffered saline (PBS) (Invitrogen).
13. Freeze medium: 50% (v/v) FCS (TCS Biologicals) and 20% (v/v) dimethyl sulfoxide (Sigma-Aldrich).
14. Cell lysis buffer: 10 m*M* Tris-HCl (pH. 8.0), 100 m*M* NaCl, 1 m*M* EDTA, 1% (w/v) SDS, and proteinase K (0.5 mg/mL).
15. Proteinase K (Roche, Lewes, UK).

### 2.3. Isolation and Analysis of ES Cell DNA

1. Isopropanol.
2. 70% (v/v) Ethanol.
3. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.

4. Restriction endonuclease enzymes and reaction buffers.
5. Optimized nylon membranes (VWR, Poole, UK), radiolabeled hybridization probes, solutions for Southern blot hybridization analysis.

### 2.4. Cre Expression in ES Cells

1. ES cells and culture media as described in **Subheading 2.2.**
2. Cre-expressing plasmid, e.g., pOG231 *(7)*.
3. HAT supplement (Sigma-Aldrich): 5 m*M* hypoxanthine, 0.02 m*M* aminopterin, and 0.8 m*M* thymidine.

## 3. Methods

The methods outlined describe the isolation of clones containing *loxP* recombination sites (the targeting clones), the transfection of ES cells with the targeting clones, the expression of Cre recombinase in the targeted ES cells so that site-specific homologous recombination between the *loxP* sites will occur, and the selection of ES cells in which the correct recombination event has occurred.

### 3.1. Isolation and Characterization of Genomic Clones for Subsequent Targeting of loxP Sites to the Mouse Genome

#### 3.1.1. Screening of 5' and 3' hprt Libraries

The use of two mouse genomic DNA libraries called the 5' and 3' *hprt* genomic libraries *(6)* greatly simplified the production of targeting clones. The libraries were constructed using vectors that contain *loxP* sites and selectable markers, and they represent most of the mouse genome cloned as fragments of about 7.5–8.5 kb. The vectors used to prepare the two libraries differ in their selectable markers and the *HPRT* gene fragments that they contain (*see* **Fig. 1A**). The vector for the 5' *hprt* library contains exons 1 and 2 of the *HPRT* gene, a neomycin resistance gene, and a *loxP* site. The 3' *hprt* library vector contains intron 2 and exons 3–9 of the *HPRT* gene, the puromycin resistance gene, and a *loxP* site *(6)*. **Figure 1A** shows the arrangement of a cluster of *Fmo* genes on mouse chromosome 1. To delete the *Fmo1, Fmo2,* and *Fmo4* genes it was necessary to isolate a clone corresponding to the 5' end of *Fmo2* and another corresponding to the 3' end of *Fmo4* (*see* **Note 3**). This enabled us to target *loxP* sites to flank the *Fmo4, Fmo1* and *Fmo2* gene cluster (*see* **Note 4**).

1. Inoculate a culture of XL 1-Blue MRA cells into NZCYM broth supplemented with maltose and magnesium (*see* **Note 5**). Grow to an optical density of approx 0.5.
2. Melt NZCYM top agar and keep at 55°C.
3. Dilute each library to give 50,000 plaque-forming units (PFU)/100 µL with SM buffer and place in a Falcon tube.
4. Add 600 µL of the XL 1-Blue MRA cells (from **step 1**) to each Falcon tube. A separate tube is needed for each agar plate. Twenty such infections are needed to represent each library. Each plate should give rise to about 50,000 plaques.
5. Incubate the tubes at 37°C for 15 min with gentle shaking, and then add 6.5 mL of NZCYM top agar. Immediately pour the contents onto NZCYM agar plates.
6. Leave the plates at room temperature for 20 min to allow the agar to set. Invert the plates and incubate at 37°C overnight (*see* **Note 6**).

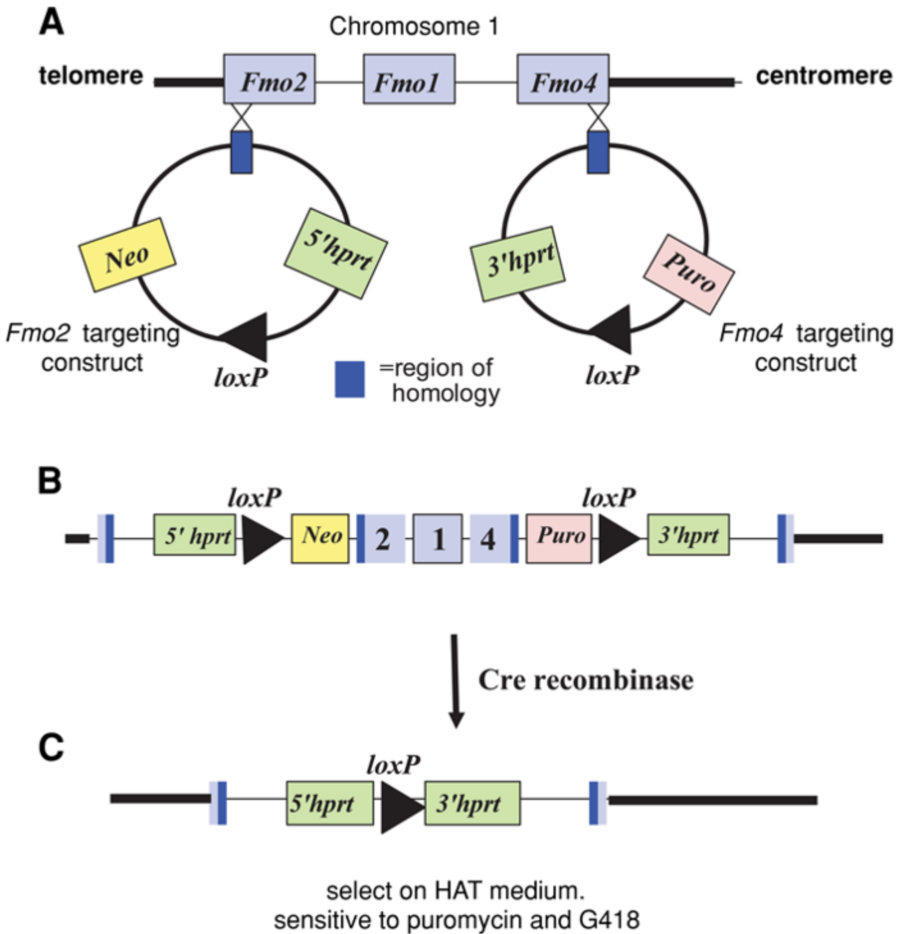


Fig. 1. Deletion of genes for *Fmo1, Fmo2,* and *Fmo4* from mouse chromosome 1. **(A)** ES cells are electroporated successively with two targeting constructs to insert *loxP* sites at either end of the genes to be deleted. **(B)** The targeting constructs integrate into chromosome 1 by homologous recombination, thus inserting two *loxP* sites, the neomycin (*neo*) and puromycin (*puro*) resistance genes and the *hprt* gene fragments, as indicated. **(C)** After Cre recombinase expression in the "doubley"-targeted ES cells, the sequences flanked by the *loxP* sites are excised and subsequently degraded in the cell. The *hprt* gene fragments are brought together and produce the HPRT protein, which allows the selection of cells in which the correct chromosomal rearrangement has occurred.

7. When plaques are visible, cool the plates to 4°C for about 2 h.
8. Place an NX nylon filter onto the surface of each plate. Use a needle dipped in Indian ink to prick holes through the filter and into the agar. These ink marks serve as future orientation marks. Remove the filter and replace with a second filter. Mark the same orientation positions as those on the first filter. Remove the filter.

9. Place each filter for 3 min on a sheet of Whatman 3MM paper presaturated in denaturation solution (DNA side up).
10. Transfer the filters for 6 min to a sheet of Whatman 3MM paper presaturated in neutralization solution.
11. Rinse the filters in 2X SSC solution and leave to air-dry on a sheet of Whatman 3MM paper.
12. Bake the filters at 80°C for 2 h or crosslink DNA to the filters by ultraviolet irradiation at 254 nm (0.15 J/$cm^2$).
13. Wet the filters in Church buffer prewarmed to 65°C. All filters to be hybridized to the same probe can be placed in the same airtight plastic container. Incubate the filters for 2 h at 65°C. The volume used should be sufficient to cover each filter.
14. Add an appropriate radiolabeled DNA fragment that will hybridize to the gene sequence to be isolated and subsequently targeted for deletion (*see* **Note 7**). Leave the filters at 65°C overnight.
15. Wash the filters in 0.1X SSC, 0.1% SDS for 1 h at 65°C, and then expose to X-ray film in sealed cassettes at –70°C.
16. Select plaques that give a hybridization signal on both replica filters. Using the wide end of a Pasteur pipet, pick an agar plug, and then immerse it in 1 mL of SM buffer and leave overnight.
17. Dilute the eluted phage particles 1/10, 1/100, and 1/1000 in SM buffer. Use 100 µL of each dilution to infect 100 µL of XL 1-Blue MRA (P2) cells for 15 min at 37°C.
18. Repeat **steps 5–16**.
19. Using the thin end of a Pasteur pipet, select plaques that give hybridization signals on replica filters.
20. Incubate the agar plug in 500 µL of SM buffer overnight (*see* **Note 8**) and store at 4°C.

#### *3.1.2. Circularization of Phage Clones Into Plasmids Using Cre-Expressing Bacteria*

This procedure is carried out to convert the bacteriophage genome into a circular molecule that will survive in *E. coli* cells as a plasmid. This procedure is possible because the libraries were constructed in phage vectors with automatic plasmid excision capabilities. The phage vectors have a plasmid backbone and two *loxP* sites that flank all the elements except the phage arms. When the phage infects a bacterial strain containing Cre recombinase, such as BNN132, the enzyme catalyzes a recombination event between the *loxP* sites, which induces plasmid excision ***(6)***.

1. Incubate a phage clone ($1 \times 10^8$ PFU) with 1 mL of a fresh overnight culture of *E. coli* BNN132 cells supplemented with 10 m*M* $MgCl_2$ for 1h at 30°C without shaking.
2. Dilute the culture twofold in LB broth and incubate for a further 1 h at 30°C in a shaking incubator. Plate cells on a 10-cm LB agar plate supplemented with ampicillin (50 µg/mL) and 0.1% glucose. Incubate overnight at 37°C.
3. Because the clones now propagate as plasmids, select colonies and inoculate LB medium containing ampicillin (50 µg/mL) (*see* **Note 9**).
4. The amount of plasmid DNA isolated from BNN132 cells is small and the DNA is difficult to digest with restriction endonucleases. Therefore, transform a strain such as DH5 α with the isolated plasmid DNA. Then prepare glycerol stocks in this strain and isolate plasmid DNA by standard methods.

### *3.1.3. Analysis of Targeting Vectors*

To confirm the insertion of DNA at the appropriate position in the mouse genome it is essential first to produce a detailed map for a set of restriction endonuclease cutting sites around the genomic target area, and to compare this with that of the targeting construct isolated as described in **Subheading 3.1.1.** The two maps should correspond for all sites within the regions of homology that they share (*see* **Note 10**).

1. Digest plasmids isolated as described in **Subheading 3.1.2.** with the selected restriction endonucleases.
2. Analyze the DNA fragments produced by agarose gel electrophoresis.
3. Blot each gel to a nylon filter. Incubate the filters for 2 h in prehybridization solution. Add the appropriate labeled DNA probe. In our experiments, this was a piece of DNA corresponding either to an exon of *Fmo2* or *Fmo4* or to a noncoding section of the genes, the position of which we had previously mapped onto the genome. Hybridize under conditions of high stringency and wash filters as described in **Subheading 3.1.1.**

The vectors used to construct the 3' and 5' *hprt* mouse genomic libraries described above contain all the necessary genetic elements required for gene targeting, chromosomal engineering and coat color tagging. Thus, the clones isolated are the insertion vectors to be used for the homologous recombination event in ES cells. However, to achieve the desired integration event, it is essential to check the orientation of the backbone elements and insert with respect to the orientation of chromosomal DNA to be deleted. For a chromosomal deletion event to be successful, the *loxP* sites from both targeting vectors (3' and 5'), once integrated into the genome, must lie in the same orientation with respect to one another. In addition, the two segments of the *HPRT* minigene must join appropriately after recombination so as to reconstitute the gene function (*see* **Note 11**). If the genomic DNA fragment is in the wrong orientation with respect to the *loxP* site, it must be flipped over as follows.

1. Remove the genomic insert from the targeting vector backbone by digestion with the enzyme *Asc*I.
2. Add DNA ligase to religate the genomic fragment back into the vector. Transform competent *E. coli* cells and plate on LB agar plates containing ampicillin (50 µg/mL).
3. Pick 20 colonies, isolate plasmid DNA, and check the orientation of the DNA insert, using appropriate restriction endonucleases to identify a clone that is in the correct orientation for targeting to the mouse genome.

### *3.1.4. Linearization of Targeting Vectors*

1. Inoculate a single colony (from **Subheading 3.1.2., step 4** or **Subheading 3.1.1., step 3**) into LB medium containing ampicillin (50 µg/mL).
2. Isolate plasmid DNA from overnight cultures using standard methods.
3. Linearize the DNA by digestion with an appropriate restriction enzyme (*see* **Note 12**).
4. Add an equal volume of a 1:1 mix of buffered phenol and chloroform. Vortex and remove the upper layer to a clean tube.
5. Precipitate the DNA with 2 vol of ethanol. Recover the pellet by centrifuging at 14,000*g*.
6. Air-dry the DNA pellet and resuspend in PBS at a concentration of 1 mg/mL.

### 3.2. Transfection of ES Cells With Targeting Constructs

The next step necessary to knockout the desired genes is to introduce the targeting construct, isolated as described in **Subheading 3.1.**, into the genome of an ES cell line. We used the EI4Tg2a ES cell line in our experiment (*see* **Note 13**). These cells were isolated from the mouse strain 129/Ola *(8)* and are *Hprt* deficient. They will die in HAT medium unless they have been transfected with an *HPRT* transgene (*see* **Note 14**). We used a clone isolated from the 5' *hprt* library to target the *Fmo2* gene and one that we had constructed *de novo*, by insertion of an appropriate fragment of mouse genomic DNA into the 3' *hprt* vector backbone, to target the *Fmo4* gene. The method describes the steps involved in transfecting the ES cell line with either targeting clone. In practice this is a two-step process. We first targeted the *Fmo2* gene and selected cells in which this targeting event had occurred. These cells were G418 resistant. We then transfected an ES cell line targeted at the *Fmo2* locus with the *Fmo4* targeting construct. Cells that have incorporated the second targeting construct are G418 and puromycin resistant.

1. Culture cells at 37°C in 5% $CO_2$ on gelatin-coated 6-cm plastic tissue culture plates in 4 mL of supplemented KO DMEM. At least 10 such plates are required. Split the cells 1 into 5 2 d before transfection.
2. Wash the cells with PBS, add 0.5 mL of trypsin solution to each plate, and incubate at 37°C for 3 min. Cells should detach easily when the plate is tapped. Collect cells from each plate with 1 mL of fresh medium using a Pasteur pipet. Ensure that the cells are resuspended by passing them through a pipet repeatedly. Transfer the cell suspension to a 50-mL centrifuge tube and fill with fresh medium. Count the cells using a hemocytometer (*see* **Note 15**).
3. Centrifuge the cells at 2000*g* and resuspend in PBS so as to give a density of $5 \times 10^7$ cells/mL.
4. Electroporate $5 \times 10^7$ of the suspended ES cells with 50 µg of linearized targeting construct (*see* **Subheading 3.1.4.**) in a 0.4-cm-wide cuvet. We used a Bio-Rad electroporator set at 800 V, 3 µF for 0.1 s.
5. Plate the electroporated cells at a density of $5 \times 10^6$ cells/10-cm dish (10 mL of medium/dish.)
6. After 24 h, add fresh medium containing either G418 (400 µg/mL), for cells targeted with clones from the 5' *hprt* library, or puromycin (0.4 µg/mL), for cells targeted with clones from the 3' *hprt* library.
7. Using a stereo dissecting microscope, pick individual colonies into 96-well plates as soon as they are visible (usually 10 d) (*see* **Note 16**).
8. Grow the cells to confluency (about 5 d).
9. Remove the culture medium and wash the cells in PBS. Add 30 µL of trypsin solution to each well. Split each plate into four replica 96-well plates. To two of the plates add 100 µL of freeze medium to each well, and freeze the plates at –70°C.
10. To the remaining two plates add 100 µL of lysis buffer to each well. Incubate the plates overnight at 37°C. Store these plates at –20°C; they will be used to extract genomic DNA (*see* **Subheading 3.3.**).

### 3.3. Selection of Correctly Targeted ES Cells

This method requires the extraction and characterization of DNA from about 500 colonies (for targeting of the *Fmo2* gene) and about 1000 colonies (for targeting of the *Fmo4* gene) (*see* **Note 17**). The isolated DNA from each ES cell clone is analyzed by

Southern blotting with probes that identify a correct homologous recombination event. The DNA probes used must hybridize to fragments of a different size in DNA isolated from cells in which the correct targeting event has occurred from that in DNA isolated from cells in which a random integration event, or nothing, has occurred.

1. Process one plate at a time. Thaw the plate of lysed cells (*see* **Subheading 3.2.**) at 37°C.
2. Transfer each lysate from a well of the plate to a 1.5-mL Eppendorf tube (*see* **Note 18**).
3. To precipitate the ES cell genomic DNA, add 130 μL of isopropanol to each tube. Mix contents by inverting the tubes four to five times.
4. Pellet the DNA by centrifuging at 14,000*g* in an Eppendorf benchtop centrifuge for 10 min at 4°C.
5. Discard the supernatant and, to the pellet, add 50 μL of 70% ethanol. Invert the tubes four to five times, and then centrifuge as in **step 4** for 5 min. Remove the ethanol and add 50 μL of TE. Incubate the samples at 65°C for at least 2 h to resuspend the DNA. The DNA is now ready for digestion with the appropriate restriction enzymes.
6. Analyze the DNA from 96 samples at a time. Transfer the DNA (5–10 μg) from each of 96 ES cell clones from Eppendorf tubes into individual wells of a 96-well microtiter plate.
7. Digest the DNA in a 40-μL reaction volume. Prepare a master mix of reaction buffer, BSA, water, and enzyme such that each DNA sample contains lX reaction buffer, 0.4 μg of BSA, and 25 U of the appropriate enzyme (*see* **Note 19**). Incubate the plate at 37°C for 6 to 7 h.
8. Electrophorese the DNA through 0.8% agarose gels at 60 V overnight (*see* **Note 20**).
9. Blot the gels to optimized nylon membranes. Hybridize under high-stringency conditions and wash as described in **Subheading 3.1.1.** After analysis of hybridizing fragments, it may be necessary to digest the DNA with a second enzyme to confirm that the correct targeting event has occurred.

### 3.4. Cre Expression in ES Cells

ES cells that have been correctly targeted at both loci can then be transfected with a plasmid expressing the enzyme Cre recombinase. This enzyme catalyzes a recombination reaction between any pair of *loxP* sites. The end product of the recombination event will depend on the position of the *loxP* sites in the genome (in *cis* or *trans*) and the relative orientation of the *loxP* sites with respect to one another and to the centromere. For a deletion of the sequence between the targeted loci, the *loxP* sites must be in *cis* (i.e., on the same chromosome) and in the same orientation *(9)*. It is not possible to determine whether the two targeting events have occurred in *cis* or in *trans* in any given cell clone; therefore, it is necessary to transfect the Cre-expressing plasmid in a few double-targeted clones to be sure to obtain the desired chromosome rearrangement *(9)*.

1. Grow three or four individual double-targeted ES clones as described in **Subheading 3.2.**
2. Trypsinize the cells, wash, and resuspend them in PBS at a density of $5 \times 10^7$ cells/mL.
3. Electroporate $5 \times 10^7$ of the suspended ES cells with 50 μg of supercoiled Cre-expressing plasmid pOG in a 0.4-cm-wide cuvet. We used a Bio-Rad electroporator set at 800 V, 3 μF for 0.1 s.
4. Plate the electroporated cells at a density of $5 \times 10^6$ cells/10-cm dish.
5. After 24 h, replace the medium with fresh medium containing HAT.

### 3.5. Selection and Screening of Deleted ES Cell Lines

Once cells have been selected for the presence of the *HPRT* minigene (using HAT), a simple way of confirming that the deletion has occurred is to select the clones again in both G418 and puromycin. Because of the way in which the deletion occurs both the neomycin and the puromycin resistance genes are deleted along with the chromosome fragment, and, therefore, cells that have the deletion should again be sensitive to both of these drugs but be resistant to HAT.

1. Using a stereo dissecting microscope, pick individual colonies into 96-well plates as soon as they are visible (usually 10 d).
2. Grow the cells to confluency (about 5 d). Remove the culture medium and wash the cells in PBS. Add 30 µL of trypsin solution to each well. Split each plate into four replica 96-well plates. To one plate, add 100 µL of freeze medium to each well and freeze the plate at –70°C.
3. Treat each of the remaining three plates with one of the three selection drugs: G418, puromycin, or HAT. Allow the cells to grow (or die) in the selection media for about 1 wk.
4. Thaw cell clones that are HAT resistant but puromycin and G418 sensitive from the frozen master plate and grow to confluency. Then split and make frozen stocks ready for further analysis.
5. A proportion of all clones obtained that are $HAT^{RES}/puro^{SEN}/G418^{SEN}$ can then be grown and analyzed by both Southern blotting and fluorescence *in situ* hybridization (*see* Chapter 35).
6. For Southern blotting, process the cells as described in **Subheading 3.3.** The restriction enzyme used will depend on the probe chosen so that it detects a different restriction fragment from the genome of cells carrying a deletion compared with that obtained from the genome of WT cells.

## 4. Notes

1. The majority of clones from both the 5' and the 3' library have now been sequenced and mapped at the Wellcome Trust Sanger Institute. They can be viewed at www.ensembl.org/Mus_musculus as DAS sources under the heading "MICER clones." Once a clone that contains a sequence that maps to an appropriate location has been identified, it can be ordered from the Wellcome Trust Sanger Institute directly without the need to screen the library. However, not all clones have been sequenced, and not all genomic regions are represented in the library; therefore, sometimes these or other genomic libraries will have to be screened in order to select sequences necessary for the construction of appropriate targeting vectors.
2. Targeting clones can be grown in large scale and DNA purified either by CsCl gradient centrifugation or by using a commercial kit such as that provided by Qiagen (Crawley UK).
3. It was not possible to isolate a genomic clone containing *Fmo4* sequences from the *hprt* library. Instead, we selected a random clone from this library and released the DNA insert by digestion with *Asc*I. The fragments were separated by agarose gel electrophoresis. The vector fragment was recovered and dephosphorylated. A previously cloned 8.2-kb *Hin*dIII fragment of the *Fmo4* gene was released from the vector pBluescript by digestion with *Bss*HII. This enzyme produces ends that are compatible with those produced by *Asc*I. The *Bss*HII-*Fmo4* fragment was ligated into the *Asc*I site of the targeting vector and the resulting recombinant clone used for the transfection of ES cells.

4. The order of the genes in the *Fmo* gene cluster is as follows: centromere- *Fmo4-Fmo1-Fmo2*-telomere. The genes are transcribed from telomere to centromere so that the most centromeric part of the cluster is at the 3' end of the *Fmo4* gene and the most telomeric part is at the 5' end of the *Fmo2* gene.
5. To obtain sufficient plaques, it is necessary to use broth supplemented with maltose and magnesium. Without these supplements the plaques obtained will be few and small.
6. The optimum period for plaque formation was found to be about 12 h. This may be an inconvenient time, and if it is not possible to leave the plates at 37°C for the full 12 h then remove the plates from the incubator and leave overnight at room temperature.
7. Any part of the gene that is to be targeted can be used as a probe. In practice, only cDNA or exon sequences are used because they are generally free from repeat sequences (which must not be used as probes). If one wishes to target specifically the 5' or the 3' end of the gene, it makes more sense to use a fragment of the gene corresponding to the desired area to be targeted. The length of the probe is not too important, but ideal probes are around 1 kb. Reverse transcriptase polymerase chain reaction (PCR) products make ideal probes for screening libraries. However, before using a probe to screen a library, it is advisable to ensure, by Southern blot hybridization to genomic DNA, that it gives a single, clean band. In our experiments, we used a full-length mouse FMO2 cDNA and a PCR product corresponding to exon 6 of the *Fmo4* gene.
8. If it is not possible to pick single positive plaques at this stage, an agar plug containing more than one plaque can be soaked in buffer as before. Rounds of screening must be repeated until single positive plaques are isolated.
9. Colonies formed by BNN132 are much smaller than those obtained with most other *E. coli* strains.
10. It is now possible to download complete genomic sequences from the mouse genome database (www.ensembl.org/Mus_musculus) and to analyze these for restriction endonuclease sites. The results can be compared with those derived from experimental data. However, it is essential to bear in mind that there are likely to be discrepancies both in the number of cutting sites and in the sizes of the fragments derived between one's data and those from a genome project. These differences are owing to mouse strain differences as well as sequencing errors. The genome databases are useful as frameworks to work from, but they are *not* a substitute for an experimentally derived restriction map of a genomic clone.
11. Other types of rearrangements such as inversions and translocations require different orientations of the *loxP* sites ***(9)***.
12. DNA to be integrated into the ES cell genome must be linearized by digestion at a single restriction site located within the region of targeting construct that is homologous to the gene to be targeted. Preferably, this site should be located in the middle of the region of homology in the clone. The restriction endonuclease used must therefore not cut the vector backbone (a full list of enzymes that cut the vector backbone can be found at www.ensembl.org). Potentially suitable enzymes identified by analysis *in silico* must be tested empirically to confirm their suitability.
13. Many ES cell lines are available and most require LIF as a supplement in the growth medium. Some lines, such as E14Tg2a, can be grown on gelatin-coated plates without a feeder layer, but others, such as D3, must be grown on feeder layers. It is important to know the mouse strain from which the ES cells were derived because this will determine the most compatible DNA library from which to obtain genomic clones. The frequency of homologous recombination is greater if the region of homology cloned into the vector

DNA is isogenic to that of the ES cell genomic DNA. Most mouse genomic libraries (and the libraries described in this chapter) have been made from DNA isolated from 129 mice.

14. The *HPRT* gene fragments included in the 5' and 3' targeting vectors are of human origin.
15. Count all cells in 16 squares in the hemocytometer. The number of cells X$10^4$ gives the number of cells/milliliter of suspension.
16. To pick colonies, aspirate the medium from the plates, wash the cells with 5 mL of PBS, aspirate and discard, and then add 10 mL of PBS to the cells. Visualize the colony under the stereo dissecting microscope, and using a Gilson P200 pipet set at 20 µL, aspirate the colony into the plastic pipet tip, and then transfer to a well in a 96-well plate containing 30 µL of trypsin solution. Process 24 colonies at a time. Then incubate the plate at 37°C for 3 min, and transfer the colonies to a fresh 96-well plate containing 150 µL of supplemented medium.
17. As many colonies as possible should be picked. However, there is little point in picking more than about 1000 because the downstream processing is too laborious for more than this number. If no homologous recombinant cells have been identified when 500 cells have been analyzed, it is best to start again with a different targeting construct.
18. Transfer of lysed ES cells from the wells of the microtiter plate to 1.5-mL Eppendorf tubes is laborious. However, subsequent isolation of the DNA in the tubes is more efficient than in the plate. In our hands, methods that invert the plate at various stages, to drain off liquid during the DNA isolation, lead to the loss of too many samples and to lower DNA yields.
19. We used *Sac*I for the *Fmo2*-targeted clones and *Bam*HI and *Eco*RV for the *Fmo4*-targeted clones. The reaction buffer is as recommended by the supplier of the enzyme. A multichannel pipettor is convenient to use for dispensing of the enzyme reaction mix.
20. It is essential that the DNA fragments be sufficiently separated for subsequent analysis of hybridizing fragments.

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# Characterization of Targeted Mouse Embryonic Stem Cell Chromosomes

## *Karyotyping and Fluorescence* In Situ *Hybridization of Metaphase Spreads*

**Diana Hernandez, Ian R. Phillips, and Elizabeth A. Shephard**

**Summary**

The manipulation of genes in mouse embryonic stem (ES) cells can result in chromosome abnormalities. This chapter describes methods for karyotyping of the manipulated ES cell line before injection into blastocysts and the use of fluorescence *in situ* hybridization to confirm the deletion of a targeted gene. The method is illustrated by describing how an ES cell line targeted for the deletion of *Fmo* genes was characterized.

**Key Words:** Flavin-containing monooxygenase genes; *Fmo1*; *Fmo2*; *Fmo4*; karyotyping; embryonic stem cells; gene deletion; chromosome 1.

## 1. Introduction

The chromosome manipulation events used to delete the *Fmo1*, *Fmo2,* and *Fmo4* genes from the genome of mouse embryonic stem (ES) cells are described in Chapter 34. These genes are clustered on mouse chromosome 1 ***(1)***. Before injection of the manipulated ES cells into mouse blastocysts, it is necessary to determine that the normal mouse karyotype of 40 chromosomes (19 pairs of autosomes and X and Y) has been maintained in these cells. Targeted ES cell clones shown to carry the normal complement of mouse chromosomes are subsequently analyzed by fluorescence *in situ* hybridization (FISH) ***(2)*** to confirm that the appropriate chromosomal deletion has occurred. It is necessary to identify ES cells that are heterozygous for the deletion; that is, the targeting and deletion event has occurred on only one of a pair of chromosomes. These are the cells that will be used to produce chimeric mice heterozygous for the gene deletion (*see* Chapter 36).

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## 2. Materials

### 2.1. Preparation of Metaphase Chromosome Spreads

1. Culture of ES cells carrying the desired gene deletion, as described in Chapter 34, **Subheading 3.5.**).
2. Knockout (KO) Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Paisley, UK) supplemented with 15% fetal calf serum (Foetal Calf Serum [European Origin]); TCS Biologicals, Buckingham, UK), 0.1 m*M* 2-mercaptoethanol (Sigma-Aldrich, Poole, UK), penicillin (50 U/mL), streptomycim (50 mg/mL), 1 m*M* L-glutamine (all from Gibco-BRL/Invitrogen), and ESGRO leukemia-inhibiting factor (1000 U/mL) (Cemicon Europe, Chandlers Ford, UK).
3. Tissue-culture dishes treated with 0.1% gelatin (Sigma-Aldrich).
4. Trypsin solution: 1.25 g of porcine trypsin powder (Invitrogen), 0.2 g of EDTA, 3.5 g of NaCl, 0.595 g of $Na_2HPO_4$, 0.12 g of $KH_2PO_4$, 0.185 g of KCl, 0.5 g of D-glucose, 1.5 g of Tris, and 0.5 mL of phenol red (Sigma-Aldrich). Make up to 500 mL with tissue-culture-grade water. Adjust the pH to 7.6 with HCl. Filter-sterilize using a 0.2-µm filter and store in aliquots at –20°C.
5. Colcemid (Invitrogen).
6. Phosphate-buffered saline (PBS) (Invitrogen).
7. Hypotonic solution: 0.56% KCl.
8. Fixative: ice-cold methanol:glacial acetic acid (3:1 [v/v]).
9. Falcon tubes (15 mL) (BD BioSciences Clontech, Oxford, UK).
10. Glass microscope slides and cover slips.

### 2.2. Karyotyping of Metaphase Chromosome Spreads

1. Slides of metaphase chromosome spreads prepared as in **Subheading 3.1.**
2. Giemsa's stain/Gurr (BDH): 10% (v/v) in water.
3. Xylene-based mountant solution.
4. Cover slips, light microscope.

### 2.3. FISH of Metaphase Chromosome Spreads

#### 2.3.1. Labeling of Probe

1. Metaphase chromosome spreads prepared as in **Subheading 3.1.**
2. Gene fragment to be labeled.
3. BioNick DNA labeling system (Invitrogen).
4. ProbeQuant G–50 microcolumns (Amersham Bioscience).
5. TE: 10 m*M* Tris-HCl (pH 8.0), 1 m*M* EDTA.
6. Appropriate labeled mouse whole chromosome paints (STAR*FISH chromosome paints) (Cambio, Cambridge, UK).
7. Salmon sperm DNA and mouse Cot1 DNA (Invitrogen).
8. Absolute ethanol (VWR International, Poole, UK).
9. 3 *M* Sodium acetate, pH 5.5.
10. 20X Saline sodium citrate (SSC): 175.3 g of NaCl, 88.2 g of sodium citrate made up to 1 L with deionized water.
11. Hybridization solution: 50% (v/v) formamide, 2X SSC, and 10% (w/v) dextran sulfate.

#### 2.3.2. Preparation of Slides for Hybridization

1. Dehydration alcohol series: 70, 90, and 100% ethanol.
2. RNase A (1 mg/mL) (Roche, Lewes, UK).

3. 2X SSC: dilute 20X SSC (**Subheading 2.3.1., item 10**) in water.
4. PK buffer: 200 m*M* Tris-HCl (pH 7.4), 20 m*M* $CaCl_2$.
5. Proteinase K (50 μg/mL) (VWR International).
6. PBS (Invitrogen).
7. 2% Paraformaldehyde (v/v) (VWR International) in PBS.
8. Denaturing solution: 70% (v/v) formamide, 2X SSC.
9. Coplin jars, humid chamber, oven, glass cover slips, 37°C water bath.

#### *2.3.3.* In Situ *Hybridization*

1. 50% (v/v) Formamide in 2X SSC.
2. FixoGum rubber cement (Qbiogene, Cambridge UK)
3. 2X SSC.
4. 4X SSC, 0.3% (v/v) Tween-20.
5. Blocking solution: 5% nonfat milk powder in 4X SSC.
6. Avidin fluorescein isothiocyanate (FITC) solution: 5 μL of fluorescein avidin DCS (cell-sorter grade fluorescein avidin D) (2 mg/mL) (Vector, Burlingame, CA) in 1 mL of blocking solution.
7. Biotinylated antiavidin solution: 10 μL of biotinylated antiavidin D (0.5 mg/mL) (Vector Labs Inc.) in 1 mL of blocking solution.
8. PBS (Invitrogen).
9. Dehydration alcohol series (*see* **Subheading 2.3.2., item 1**).
10. Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector).
11. Fluorescence microscope and cooled charge-coupled device (CCD) camera.

## 3. Methods

### *3.1. Preparation of Metaphase Chromosome Spreads* (3)

1. Grow ES cells to confluency (about 5 d) at 37°C in 5% $CO_2$ on gelatin-coated 6-cm plastic dishes in 4 mL of supplemented KO DMEM.
2. Trypsinize the cells (*see* Chapter 34, **Subheading 3.2, step 2**).
3. Split each dish one into five fresh dishes. Add 4 mL of supplemented KO DMEM and leave the cells to grow overnight.
4. The next day, carefully remove the medium from the cells and replace with fresh medium. Allow the cells to grow for 2 h.
5. To each dish add colcemid to a final concentration of 0.4 μg/mL. Leave the cells to arrest for about 1 h.
6. Trypsinize the cells as in **step 2** and place the cells from each plate into separate 15-mL Falcon tubes.
7. Pellet the cells by centrifuging at medium speed in a benchtop centrifuge for 5 min. We use a Heraeus Biofuge primo tabletop centrifuge set at 3000 rpm (~4000*g*).
8. Carefully remove the supernatant and resuspend the cells in 5 mL of PBS. Pellet the cells by centrifuging as described in **step 7**.
9. Resuspend each pellet in a few drops of PBS. Add 5 mL of hypotonic solution. Mix gently and allow the cells to swell at 37°C for 5 min.
10. Pellet the cells carefully by low-speed centrifugation (600*g*) for 5 min. Aspirate most of the supernatant, leaving just a few drops of liquid in the tube. Carefully resuspend the pellet in this liquid.
11. Add 5 mL of fresh, ice-cold fixative solution a drop at a time. Continually flick the tube to mix the cell suspension (*see* **Note 1**).
12. Leave the cells to stand at room temperature for 5 min. Centrifuge at low speed for 5 min.

13. Repeat **steps 11** and **12** twice.
14. Resuspend the final pellet in 1mL of fixative (*see* **Note 2**).
15. Using a pipet, from a height of 60–80 cm, drop the cells onto a clean microscope glass slide (*see* **Note 3**). Use two drops per slide. Do not allow the two drops to merge. Allow to dry.
16. Inspect the chromosomes with a phase-contrast microscope. Several metaphase spreads should be seen on each slide (see **Note 4**).
17. Allow the slides to dry at room temperature overnight.

### 3.2. Karyotyping of Metaphase Chromosome Spreads

Only ES cell lines in which the normal mouse karyotype of 40 chromosomes has been preserved should be used to inject into mouse blastocysts. The ES cell line (*see* Chapter 34, **Subheading 3.2.**) is of male origin ***(4)***.

1. Incubate slides of metaphase chromosome spreads for 20 min in Giemsa's stain.
2. Rinse thoroughly in water and leave to dry overnight at room temperature.
3. Mount a cover slip over the stained spreads using Permount or a similar mounting medium.
4. View slides under a light microscope using a ×100 oil immersion lens.
5. Count the chromosomes in a minimum of 20–30 spreads (*see* **Note 5**).

### 3.3. FISH of Metaphase Chromosome Spreads

Metaphase chromosome spreads prepared as described in **Subheading 3.1.** are hybridized to suitably labeled probes to ensure that the deletion event has occurred on only one of the pair of chromosomes. **Figure 1** shows the FISH of a metaphase chromosome spread from ES cells targeted for the deletion of the *Fmo1*, *Fmo2,* and *Fmo4* genes. To aid in the identification of individual mouse chromosomes, it is helpful to use commercially available, whole chromosome paints to identify the chromosome on which the deletion event should have occurred. In **Fig. 1** mouse Chromosome 1 has been painted with a whole mouse chromosome paint labeled with Cy3 (red) and the chromosomes were hybridized to a biotin-labeled *Fmo1* gene fragment, which was subsequently detected by incubation with fluorescein-conjugated avidin. The procedure is essentially as described by Lichter et al. ***(5)*** with some modifications.

#### 3.3.1. Labeling of Probe

The specific gene probe to be used is labeled indirectly with a fluorescent dye. The color of this dye should be different from the dye used in the chromosome paint. We used a Chromosome 1 paint labeled with Cy3 (red) and a gene probe labeled with biotin, which we subsequently detected with fluorescein-conjugated avidin (green) (*see* **Note 6**).

1. Label 2 µg of the appropriate gene probe DNA in a total volume of 20 µL with biotin using a BioNick labeling system according to the manufacturer's recommendations (*see* **Note 7**).
2. Remove unincorporated nucleotides by passing the labeling reaction mixture through a ProbeQuant G-50 microcolumn (Amersham Bioscience), according to the manufacturer's recommendations and eluting it in 400 µL of TE.
3. To the labeled probe, add 10 µg of salmon sperm DNA and 10 µg of mouse Cot1 DNA. Precipitate the DNA using 1/10 vol of 3 *M* sodium acetate and 2 vol of absolute ethanol. Pellet the DNA by centrifuging at 14,000*g* for 10 min at 4°C. Pour off the supernatant. Wash the pellet in 70% ethanol and air-dry.

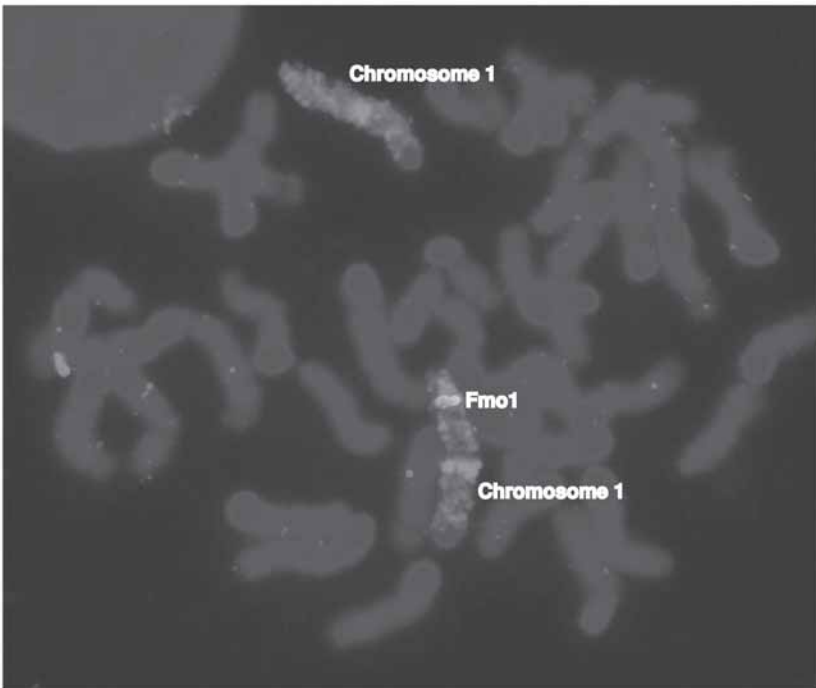


Fig. 1. Metaphase spread of mouse chromosomes. Chromosomes were hybridized with a probe for *Fmo1* and a chromosome paint specific for Chromosome 1. The two copies of chromosome 1 are visible. The signal for the *Fmo1* gene is present on only one of the two copies of chromosome 1, indicating that the ES cell line is heterozygous for a deletion of this gene.

4. Resuspend the labeled probe in 20 µL of hybridization solution.
5. Denature the probe by heating to 70°C for 5 min; then allow to renature slowly at 37°C for at least 30 min to mask any repeat sequences (*see* **Note 8**).
6. Prepare mouse whole chromosome paints for hybridization according to the manufacturer's recommendations (Cambio).

### 3.3.2. Preparation of Slides for Hybridization

1. Dehydrate metaphase chromosome spreads prepared as described in **Subheading 3.1.**, in an alcohol series. Place slides in a Coplin jar containing 70% ethanol for 5 min. Transfer the slides to a second Coplin jar containing 90% ethanol and incubate for 5 min. Transfer the slides to a third jar containing 100% ethanol and incubate for 5 min. Remove the slides and allow to air-dry.
2. To each slide add 200 µL of RNase A (1 mg/mL). Cover with a glass cover slip and incubate the slides in a humid chamber at 37°C for 1 h (*see* **Note 9**).
3. Remove the slides from the humid chamber and take off the cover slips. Immediately submerge the slides in a Coplin jar filled with 2X SSC. Rinse briefly and discard the solution. Repeat this step four more times.
4. Dehydrate the slides as described in **step 1**.

5. Immerse the slides in a Coplin jar containing PK buffer and incubate at 37°C for 10 min.
6. Transfer the slides to a second Coplin jar containing PK buffer with 35 μL of proteinase K, and incubate at 37°C for 7 min.
7. Wash the slides in PBS for 5 min.
8. Fix the slides in 2% (v/v) paraformaldehyde in PBS for 10 min at room temperature.
9. Wash the slides in PBS with shaking for 10 min at room temperature.
10. Dehydrate the slides as described in **step 1**.
11. Allow the slides to dry.
12. Add 100 μL of denaturing solution to each slide and cover with a cover slip. Place the slides in a dry oven at 75°C for 30 s.
13. Remove the cover slips.
14. Immerse the slides in a Coplin jar of ice-cold 70% ethanol.
15. Dehydrate the slides as described in **step 1**.

### *3.3.3.* In Situ *Hybridization*

1. Add 20 μL of probe in hybridization solution and 12 μL of chromosome paint to each slide. Place a cover slip carefully over the solution and seal with rubber cement. Place the slides in a humid chamber at 37°C overnight.
2. The next day remove the cover slips (*see* **Note 10**) and wash the slides in 50% (v/v) formamide in 2X SSC for 5 min at 42°C. Repeat this wash step three times.
3. Wash the slides in 2X SSC for 2 min at 42°C. Repeat this step five times.
4. Wash the slides in 4X SSC, 0.3% Tween-20 for 5 min at room temperature.
5. Incubate the slides with shaking in blocking solution for 20 min at room temperature.
6. To each slide add 100 μL of avidin FITC solution, cover with a glass cover slip, and incubate in a humid chamber at 37°C for 30 min.
7. Remove cover slips and wash slides in 4X SSC, 0.3% Tween-20 for 5 min at room temperature in the dark with shaking. Repeat this step three times.
8. To each slide add 100 μL of biotinylated anti-avidin solution, cover with a glass cover slip and incubate in a humid chamber at 37°C for 30 min.
9. Remove the cover slips and wash the slides, in the dark with shaking, in 4X SSC, 0.3% Tween-20, for 5 min at room temperature. Repeat this step three times.
10. To each slide add 100 μL of avidin FITC solution, cover with a glass cover slip and incubate in a humid chamber at 37°C for 30 min.
11. Remove cover slips and wash slides, in the dark with shaking, in 4X SSC, 0.3% Tween-20, for 5 min at room temperature. Repeat this step three times.
12. Wash slides, in the dark with shaking, in PBS for 5 min at room temperature. Repeat this step twice.
13. Dehydrate the slides as described in **Subheading 3.3.2., step 1**.
14. Mount the cover slips onto the dry slides using Vectashield mounting medium with DAPI.
15. Analyze the signals by fluorescence microscopy and image by a cooled CCD camera (Photometrics) and Smartcapture software (Vysis).

## 4. Notes

1. Do not rush this step. To avoid clumping of cells, it is essential that fixative be added slowly and dropwise and with constant gentle mixing.
2. Pellets can be stored in fixative at –20°C indefinitely.
3. The slides should be cleaned with ethanol and removed from a humidified container just before use. The humidity enhances the adherence of the chromosomes to the glass.
4. If few metaphase chromosomes are observed, repeat the procedure in **Subheading 3.1.**

Try varying the length of time the cells are treated with colcemid or the time cells are cultured before the addition of colcemid. Often different clones of the same cell line behave differently. Different clones might require slightly different culture times to obtain reasonable numbers of metaphase chromosome spreads. Many metaphase spreads should be available to score, because not all of the metaphase spreads prepared will have a full set of chromosomes, and not all of the spreads will hybridize optimally. It is essential that when inspected under a phase-contrast microscope at low resolution more than 10 metaphase spreads can be seen in a given field of vision.

5. Only count spreads in which chromosomes can be distinguished unambiguously and where there are no, or few, overlapping chromosomes. Spreads in which chromosomes are too far apart should not be counted. Typically, a cell clone is considered to have preserved its karyotype if >70% of the metaphase spreads have 40 chromosomes.
6. Probes can be labeled directly with fluorescent dyes such as fluorescein or rhodamine using commercially available kits. We found that direct labeling works better on large probes, such as P1 artificial chromosome clones (PACs), but for smaller probes, such as phage clones, labeling with biotin and subsequently detecting the signal with a sandwich technique using fluorescein-conjugated avidin amplified the signal significantly.
7. For *in situ* hybridization, larger probes work better and whole cosmids, PACs and phage clones can be used. Because probes are labeled by nick translation there is no restriction on the size, and the larger the fragment the better the signal. There is also no need to remove the vector backbones, by restriction digestion and gel electrophoresis, before labeling because the background hybridization that they produce is minimal.
8. Because most probes used in FISH are large genomic fragments, they are likely to contain repeat sequences. To avoid these repeats hybridizing throughout the genome, the probe is preincubated with Cot1 DNA, so that all repeat sequences present in the probe will anneal to the cold competitor DNA, thus leaving only the unique labeled sequences available to hybridize to the chromosomes.
9. It is important not to exceed 1 h of incubation with RNase A because the integrity of the chromosomes may be destroyed.
10. The rubber cement should peel off the slide easily, but care should be taken not to damage the spreads when the cover slip is removed.

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# 36

## Microinjection of Targeted Embryonic Stem Cells and Establishment of Knockout Mouse Lines for *Fmo* Genes

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**Summary**

Methods are described for the injection of mouse embryonic stem cells, in which *Fmo* genes have been targeted to disrupt gene function, into 3.5-d-old blastocysts and the implantation of these into foster mothers. Successful injection and implantation of blastocysts will produce mice of mixed coat color (the chimera). Also described are methods to establish the success of blastocyst injection and implantation of germ-line transmission of the knockout (KO) mutation. Breeding strategies to produce congenic and isogenic KO mouse lines are outlined. Simple methods for the isolation of tail DNA, the tagging of mice, and record keeping of the line are also given.

**Key Words:** Knockout mice; mouse embryonic stem cells; blastocysts, tail DNA; mouse colony; breeding strategy.

### 1. Introduction

The production of knockout (KO) mouse lines requires that embryonic stem (ES) cells correctly targeted for the gene(s) to be "knocked out," as described in Chapter 34, be injected into 3.5-d-old blastocysts. The ES cell line used is derived from a 129 mouse strain (agouti coat color) whereas the host blastocyts are from the C57BL/6 (black coat color) line. Injected blastocysts are then transplanted into foster mothers. The success of blastocyst injection is indicated by the coat color of mice born to the foster mother: pups with a black coat color will have been derived from host blastocyst cells, whereas pups of a mixed coat color will have been derived from a mixture of the injected ES cells and host cells ***(1)***. The latter mice are said to be chimeric. Chimeric mice are then crossed with wild-type (WT) C57BL/6 mice to test for germline transmission of the mutant gene. If the germ line cells of the chimera were derived from the injected 129 ES cells, then the progeny of this cross will be agouti colored, because agouti coat color is dominant over black coat color. Such mice can

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then be tested to select those that are heterozygous for the KO mutation. Mice that carry the KO mutation are then backcrossed with WT C57BL/6 strain mice for 10 generations to produce a congenic mouse line *(2)*. Experiments on these mice can be carried out using WT C57BL/6 mice as controls, because the repeated backcrossing of the knockout line eliminates genes derived from the 129-derived ES cells. Backcrossing of the chimera to the 129 line will produce a coisogenic line within two generations *(2)*. The progeny of each backcrossing must be genotyped to identify mice that are heterozygous for the KO mutation so that they can be mated with the WT strain. When the line has been established, heterozygous mice can then be mated to produce mice that are homozygous for the "knocked-out" gene.

All procedures involving live animals are regulated in the United Kingdom by Home Office guidelines. The procedures described below should therefore be carried out only under the auspices of the appropriate Home Office license or the equivalent in other countries.

## 2. Materials

### 2.1. Microinjection of ES Cells Into Blastocysts

#### 2.1.1. Mice and Equipment for Isolation of Blastocysts

1. C57BL/6 stud adult males (about 20–30) or another suitable mouse strain depending on the ES cell line used.
2. C57BL/6 adult females (about 40–60).
3. Mating cages.
4. Stainless steel dental spatula for checking vaginal plugs.

#### 2.1.2. Foster Mothers and Equipment

1. CD1 vasectomized adult males (about 10–15) or another suitable mouse strain.
2. CD1 adult females (about 20–30).
3. Mating cages.
4. Stainless steel dental spatula for checking vaginal plugs.

#### 2.1.3. Culture of ES Cells

1. ES cell clone heterozygous for the mutation (deletion).
2. Penicillin/streptomycin/glutamine (100X), liquid (Invitrogen, Paisley, UK).
3. Nonessential amino acids (100X), liquid (Invitrogen).
4. Leukemia-inhibiting factor (LIF): ESGRO LIF (Chemicon Europe, Chandlers Ford, UK).
5. KO Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 15% fetal calf serum (FCS) (Foetal Calf Serum [European Origin]; TCS Biologicals, Buckingham, UK), 0.1 m*M* 2-mercaptoethanol, penicillin (50 U/mL), streptomycin (50 mg/mL), 1 m*M* L-glutamine, and LIF (1000 U/mL).
6. Tissue culture plates treated with 0.1% gelatin (Sigma-Aldrich, Poole, UK).
7. Trypsin solution: 1.25 g of porcine trypsin powder (Invitrogen), 0.2 g of EDTA, 3.5 g of NaCl, 0.595 g of $Na_2HPO_4$, 0.12 g of $KH_2PO_4$, 0.185 g of KCl, 0.5 g of D-glucose, 1.5 g of Tris, and 0.5 mL of phenol red (Sigma-Aldrich). Make up to 500 mL with tissue-culture-grade water. Adjust the pH to 7.6 with HCl. Filter-sterilize using a suitable 0.2-µm filter and store in aliquots at –20°C.
8. Phosphate-buffered saline (PBS) (Invitrogen).

#### 2.1.4. Recovery of Blastocysts

1. C57BL/6 females 3.5 d pregnant.
2. Dissecting scissors and forceps.
3. 70% Ethanol.
4. Tissue culture plates (6-cm).
5. Flushing medium: DMEM supplemented with 10% FCS, penicillin (50 U/mL), streptomycin (50 mg/mL), and 1 m*M* L-glutamine.
6. Syringes and needles.
7. Dissecting microscope.
8. Aspirator tube assembly (Sigma-Aldrich).
9. Borosilicate glass capillaries (225 pcs, 1.5-mm o.d., 1.17-mm i.d.) (Harvard Apparatus, Edenbridge, UK).
10. Mineral oil (Sigma-Aldrich).

#### 2.1.5. Injection of Blastocysts With ES Cells

1. Injection medium: DMEM with HEPES and no $NaHCO_3$ (ICN, Basingstoke, UK) supplemented with 10% FCS, penicillin (50 U/mL), streptomycin (50 mg/mL), and 1 m*M* L-glutamine.
2. Micromanipulators (Leica Microsystems, Milton Keynes, UK).
3. Inverted microscope (Leica Microsystems).
4. Injection and holding pipets (Eppendorf, Cambridge, UK).
5. ES cells.
6. Blastocysts (3.5-d-old mouse embryos).
7. Aspirator tube assembly (Sigma-Aldrich).
8. Glass capillaries (*see* **Subheading 2.1.4., item 9**).

#### 2.1.6. Transfer of Blastocyst Into Pseudopregnant Females (Uterine Transfers)

1. Pseudopregnant female mice.
2. Anesthetic (usually Hypnorm/Hypnovel).
3. Dissecting scissors, forceps, and clamps.
4. 70% Ethanol.
5. Embryo transfer pipets (*see* **Note 1** and **Subheading 2.1.4., item 9**).
6. Aspirator tube assembly (Sigma-Aldrich).
7. Injected blastocysts.
8. Sutures (Mersilk).
9. Clay Adams clips (VetTech Solutions, Congleton, UK).
10. Warm recovery chamber.
11. Dissection microscope with external light source.
12. Syringe needles (26 gage).

### 2.2. Genotyping Mice

#### 2.2.1. Tagging Mice

1. Clean cages clearly labeled with origin of mice: separate cages for male and female mice will be needed.
2. Ear punch.
3. Cauterizing pen.

4. Sharp pair of fine scissors.
5. Safe-lock, polymerase chain reaction (PCR)–clean 1.5-mL microtubes (Eppendorf).
6. Rack to hold tubes.
7. Permanent marker pen.

### 2.2.2. Isolation and Analysis of Tail DNA

#### 2.2.2.1. Isolation of Tail DNA Without Phenol

1. Rapid tail lysis buffer: 10 m*M* Tris-HCl, pH 8.0, 50 m*M* KCl, 2.5 m*M* $MgCl_2$, gelatin (0.1 mg/mL), 0.45% (v/v) Nonidet P40, and 0.45% (v/v) Tween-20.
2. Proteinase K (20 mg/mL) (VWR International, Poole, UK).
3. Isopropanol.
4. 70% Ethanol at –20°C.
5. TE: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.
6. Safe-lock, PCR-clean 1.5-mL microtubes (Eppendorf).
7. Heating block or water bath.

#### 2.2.2.2. Isolation of Tail DNA With Phenol

1. Phenol tail lysis buffer: 10 m*M* Tris-HCl, pH 8.0, 100 m*M* EDTA; 1% (w/v) sodium dodecyl sulfate; 200 m*M* NaCl.
2. **Items 2–7** as in **Subheading 2.2.2.1.**
3. Buffered phenol: liquefied phenol washed in Tris buffer (Fisher, Loughborough, UK).

## 3. Methods

### 3.1. Microinjection of ES Cells Into Blastocysts

The time schedule for injection of blastocysts with ES cells and their subsequent implantation into foster mothers is given in **Table 1**. The steps include mating of female mice to produce blastocysts, mating of foster mothers to vasectomized males, culture of the targeted ES cells, injection of ES cells into blastocysts, and implantation of injected blastocysts into foster mothers.

#### 3.1.1. Mice for Isolation of Blastocysts

1. Set up mouse matings 3.5 d ahead of injection using a suitable mouse strain (*see* **Note 2**).
2. Place in mating cages one C57BL/6 male with two C57BL/6 females (in estrus). About 28–30 males and 56–60 females are required to guarantee production of sufficient blastocysts.
3. The following day (early morning), check the females for vaginal plugs. Separate all females from males. In a separate cage(s), place all females with vaginal plugs. These represent those that are potentially pregnant (*see* **Note 3**).

#### 3.1.2. Foster Mothers

1. Set up matings to produce pseudopregnant foster females by mating vasectomized males with receptive females (*see* **Note 4**). A ratio of one male to two females is adequate. Fifteen males and 30 females should produce sufficient foster mothers for the implantation of injected blastocysts.
2. The following day, check for vaginal plugs and separate out females with positive signs of mating, i.e., vaginal plugs.

**Table 1**
**Time Schedule for Injection of Blastocysts With ES Cells and Their Implantation Into Foster Mothers**

| Day | Mice | ES cells |
|---|---|---|
| 1 | Set up matings between C57BL/6 males and female blastocyst donors. | Thaw ES cells. |
| 2 | Check vaginal plugs on mated females. Separate plugged females. Set up matings between vasectomized males and foster mothers. | Feed ES cells. |
| 3 | Check vaginal plugs on foster mothers. Separate plugged females. | Split ES cells 1 in 5. |
| 4 | | Feed ES cells. |
| 5 (AM) | | (1) Feed ES cells. |
| | (2) Flush blastocysts. | |
| 5 (PM) | | (3) Harvest ES cells. |
| | (4) Inject blastocysts with mutant ES cells and then transfer the injected blastocysts into pseudopregnant foster mothers. | |

### *3.1.3. Culture of ES Cells*

ES cells for injection are cultured in supplemented KO DMEM, as described in Chapter 34, **Subheading 3.2.** Cells must be in their growth phase. Only a few cells are required, the cells from one well of a 24-well plate are sufficient.

1. Two days before the injection of blastocysts, split a culture of ES cells one to five. Seeding cells into one or two wells of a 24-well plate will produce sufficient cells.
2. On the day of injection, and 2 h before the injection, remove medium from the cells and replace with fresh medium.
3. Two hours later, aspirate the medium, wash the cells with PBS, and add 200 mL of trypsin/well. Incubate for 3 min at 37°C and tap plate to dislodge the cells from the plate. Transfer the cells to a centrifuge tube using a Pasteur pipet. Ensure that the cells are fully suspended by passing them repeatedly through the pipet. Spin at 4000*g* for 5 min at room temperature in a benchtop centrifuge.
4. Resuspend the cells in 1–2 mL of injection medium, and take to the manipulation room. Cells can be kept in suspension in a centrifuge tube at room temperature for only very short periods of time (10–20 min) because they will start clumping.

### *3.1.4. Recovery of Blastocysts*

1. Take all plugged C57BL/6 females and kill by cervical dislocation or other Schedule 1 method.
2. Swab the abdomen with 70% ethanol. Make a transverse incision mid-ventrally and peel the skin toward the fore and hind limbs.

3. Make a transverse incision in the peritoneum. Pull the uterine horns, cut them away from the body, and lay them on a tissue.
4. Trim the fat tissue away from the horns, and cut the top and bottom of each horn so that embryos can be flushed out.
5. Take a syringe with 1 mL of flushing medium and a 26-gage needle and insert the needle at the top of the horn, and flush the medium through the horn gently onto a 6-cm tissue culture plate. Repeat this for all mice.
6. Place the tissue culture plate under a dissecting microscope and locate all the blastocysts (*see* **Note 5**). Recover the blastocysts using a drawn-out glass capillary attached to a mouth-controlled pipet (aspirator tube assembly). Transfer the blastocysts to a drop of medium suspended in mineral oil in a clean 6-cm tissue culture plate. Blastocysts can be kept suspended in medium in a $CO_2$ incubator at 37°C for a few hours before the injection of ES cells.

### 3.1.5. Injection of Blastocysts With ES Cells

Specialized equipment is required for the injection of ES cells into blastocysts. For details of micromanipulators and microscopes required for this procedure, refer to **ref.** ***3*** or ***4***. It is advisable to seek help from someone familiar with the equipment and the procedure before carrying out an injection for the first time, because manipulators may vary.

1. Set up the micromanipulators with the appropriate holding and injection pipets.
2. Fill the manipulating chamber with injection medium, and place both ES cells and blastocysts in the chamber by using a drawn-out mouth-controlled pipet.
3. With the injection pipet, pick up 10–15 ES cells.
4. Pick a blastocyst with the holding pipet and position it so that the intercell membrane is in focus. Insert the injection pipet into the blastocyst and release the ES cells into the blastocoelic cavity. Remove the injection pipet, and place the injected blastocyst at the top of the manipulating chamber, away from the noninjected blastocysts and ES cells.
5. Repeat **steps 2–4** until all available blastocysts have been injected.
6. Transfer the injected blastocysts into a drop of medium suspended in mineral oil, and leave in a $CO_2$ incubator at 37°C until ready for transfer.

### 3.1.6. Transfer of Blastocysts Into Pseudopregnant Females (Uterine Transfers)

1. Weigh a pseudopregnant female and anesthetize (*see* **Note 6**).
2. Prepare an embryo-transfer pipet by sucking up first mineral oil, followed by medium; then the embryos to be transferred (10 embryos/foster mother); and, finally, some more medium. Set aside until the mouse is fully anesthetized.
3. Clean the back of the mouse with 70% ethanol, and make a small transverse incision in the skin at the level of the first lumbar vertebra.
4. Gently slide the skin incision until the ovarian fat pad is visible through the peritoneum. Then make an incision through the peritoneum, grasp the fat pad with blunt forceps, and pull out through the opening so that the ovary, the oviduct, and a length of uterus are visible.
5. Stabilize the ovary outside the body by clamping it with a small clip.
6. Place the mouse under a dissecting microscope so that the oviduct is visible. Open a small hole in the oviduct using a 26-gage needle. Insert the embryo-transfer pipet into the hole

Fig. 1. Three chimera mice and a black color-coated littermate.

left by the needle and blow the embryos into it. Remove the pipet and return the ovary and uterine horn to the cavity.

7. Suture the peritoneum with an appropriate suture.
8. Close the skin with a clip.
9. Allow the mouse to recover in a warm chamber, and then return it to its cage. Signs of pregnancy can be seen 10 d after implantation.

#### *3.1.7. Selection and Breeding of Chimeric Mice*

After 18 d of gestation, the foster mothers should give birth. A proportion of the litter should be chimeric pups, i.e., derived from a blastocyst successfully injected with ES cells (*see* **Note 7**). Chimeras can be scored around 4–5 d after birth when their coat color can be seen. The extent to which they are agouti in color will be an indication of the ES cell contribution to internal tissues. However, germ-line contribution can only be ascertained by test breeding. Once male chimeras have reached reproductive age (7–8 wk), they can be mated to test for germ-line transmission of the ES cell mutation. If the ES cells used were derived from a 129 strain and the blastocysts from C57BL/6 mice, the chimeras can be test bred against C57BL/6 mice. In practice, male chimeras are mated to female C57BL/6 mice. If the first litter produces agouti coat–colored pups, then germ-line transmission has occurred (**Fig. 1**) (*see* **Note 8**). Each agouti coat-colored mouse has a 50% chance of carrying the mutated allele; therefore, the pups must be genotyped to determine whether they are heterozygous for the mutant allele or homozygous for the WT allele. Mice that are heterozygous for the KO mutation can be mated with each other to produce a first batch of homozygous mice, to examine the phenotype. Subsequently, chimeras that exhibit good germ-line transmission readily can be crossed with 129 mice to obtain a coisogenic line. The first

heterozygotes derived from the chimeras can also be backcrossed to C57BL/6 to breed the mutation onto this strain and to produce a congenic line (*see* **Note 9**).

## 3.2. Genotyping of Mice

### 3.2.1. Tagging of Mice

Different laboratories tag mice in different ways. We describe a simple method for ear punching that is quick and that makes subsequent identification of a particular mouse very easy. Ears are marked when mice are weaned (21 d after birth). At this time a small piece of tail is removed for subsequent DNA isolation (*see* **Subheading 3.2.2.**).

1. Prepare two cages per litter to be weaned and label them clearly with the name (or number) of the parents and their strain, followed by the litter number (i.e., first, second), and the sex (one cage for males, one for females).
2. Take one mouse at a time out of the rearing cage and scruff it so that it can't bite or fall. Ascertain its sex (*see* **Note 10**), and cut a small piece from the end of the tail (1–2 mm) and cauterize the cut end with a cauterizing pen. Place the piece of tail in an Eppendorf tube. Alternatively, hold the mouse on the bench and cut the tip of its tail while it is lying on the bench. Place a colored piece of paper or piece of plastic on the bench to make the cut-off tail tip clearly visible. Then place the mouse on your chest so that it faces you and, while holding firmly with one hand, punch a hole in the ear using an ear puncher. The numbering follows this sequence:
    a. Mouse 1: no ear punches.
    b. Mouse 2: one hole in left ear.
    c. Mouse 3: one hole in right ear.
    d. Mouse 4: two holes in left ear.
    e. Mouse 5: two holes in right ear (*see* **Note 11**).
3. Place the mouse in its new weaning cage, and proceed until all mice in the litter have been tagged.

### 3.2.2. Isolation and Analysis of Tail DNA

It is convenient to genotype mice by analyzing tail DNA using PCR. The PCR products should distinguish between mice that are WT and those that are heterozygous or homozygous for the knocked-out allele. The choice of primers will depend on the locus, the targeting vector, and the type of mutation introduced (*see* **Note 12**). We describe two methods for the isolation of tail DNA. The no-phenol method is suitable for smaller PCR products, whereas the phenol method is more reliable for the amplification of large DNA products.

#### 3.2.2.1. Isolation of Tail DNA Without Phenol

DNA isolated using a no-phenol method can be used to genotype, by PCR, mice in which the *Fmo1, Fmo2,* and *Fmo4* gene cluster has been deleted. **Figure 2A** shows the amplification products of the *HPRT* sequence used to distinguish WT littermates from those that are heterozygous or homozygous for the deletion of the *Fmo* gene cluster described in Chapter 34. **Figure 2B** shows the amplification products of the *Fmo1* gene. Mice that are negative for the *Fmo1* gene amplification product but positive for

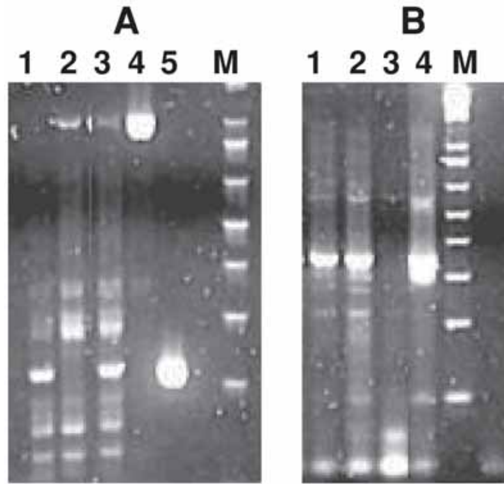


Fig. 2. Products of polymerase chain reaction (PCR) amplified by **(A)** *Hprt* or **(B)** *Fmo1* primers. **(A)** DNA was amplified from mouse tail DNA isolated from targeted mice (lanes 1–3), from a nontargeted control animal (lane 4), or from 3' *hprt* plasmid vector (lane 5). **(B)** DNA was amplified from mouse tail DNA isolated from targeted mice (lanes 1–3) and from a nontargeted control animal (lane 4). M, molecular size markers.

the *HPRT* product are homozygous for the gene deletion. Mice positive for both PCR products are heterozygous for the gene deletion, whereas mice positive for the *Fmo1* gene product and negative for the *HPRT* gene product are WT. The method described next was adapted from **ref. *5***.

1. Add 200 µL of rapid tail lysis buffer containing proteinase K to each tail tip. Flick to make sure each tail tip is covered with solution.
2. Incubate the tails for 1–18 h (*see* **Note 13**) in a heating block or water bath set at 55°C and a speed of about 850 rpm. If a heating block with a shaking facility is not available, vortex the samples occasionally.
3. Centrifuge the samples at 14,000*g* in a microcentrifuge for 60 s at room temperature.
4. Transfer 190 µL of supernatant to an Eppendorf tube containing 200 µL of isopropanol. Be sure to transcribe carefully the mouse ID onto the lid of each tube.
5. Mix the contents by inverting the tube three to four times, until a DNA thread becomes visible.
6. Centrifuge samples at 14,000g in a microcentrifuge for 5–10 min at room temperature.
7. In one movement, pour off the supernatant and then briefly touch the inverted tube edge to a tissue to drain remaining drops of liquid.
8. Add 500 µL of –20°C, 70% ethanol.
9. Centrifuge the samples at 14,000*g* in a microcentrifuge for 5 min at room temperature. If a refrigerated centrifuge is available, centrifuge the samples at 4°C.
10. In one movement, pour off the supernatant and then briefly touch the inverted tube edge to a tissue to drain remaining drops of liquid.

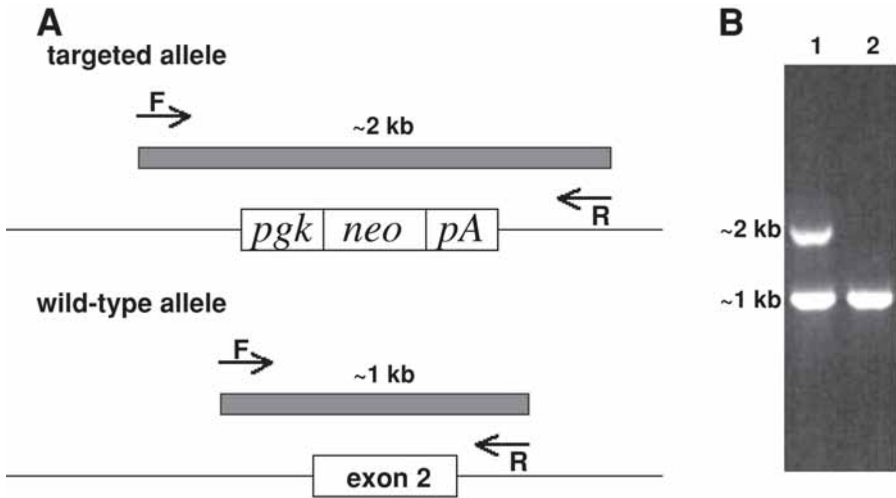


Fig. 3. **(A)** Schematic representation of polymerase chain reactino (PCR) amplification strategy used to distinguish *Fmo5* targeted allele from *Fmo5* WT allele. **(B)** Agarose gel electrophoresis of PCR products obtained using primers F and R shown in **(A)**. DNA was amplified from a chimeric mouse (lane 1) or from a WT mouse (lane 2).

11. Press the edge of a micropipet tip (e.g., a P200 tip) onto a clean tissue to bend the tip slightly. Using this modified tip, remove, with a micropipet, the remaining ethanol from the pellet, which should be clearly visible.
12. Leave the tubes with their lids open and allow the DNA to air-dry (about 10 min).
13. Add 100 µL of TE and incubate the samples at 65°C in a heating block for 15 min or at 4°C overnight, to dissolve the DNA. Store the DNA samples at 4°C. One microliter of DNA sample should contain sufficient template for a 25-µL PCR reaction.

#### 3.2.2.2. Isolation of Tail DNA With Phenol

**Figure 3** shows the simultaneous amplification of two PCR products that identify mice that are either heterozygous (>2 kb) or WT (>1 kb) for a disruption of the *Fmo5* gene. Identification of the larger amplification product requires the isolation of DNA using the phenol method described next.

1. Add 200 µL of phenol tail lysis buffer containing proteinase K to each tail tip (*see* **Subheading 3.2.1.**). Flick to make sure the tail tips are covered with solution.
2. Incubate the tails for 3–24 h (*see* **Note 13**) in a heating block or water bath set at 55°C and a speed of about 850 rpm. If a heating block with a shaking facility is not available, vortex the samples occasionally.
3. Add 300 µL of TE and 500 µL of buffered phenol. Make sure that the tubes are firmly closed. Vortex the samples. Check to ensure that no phenol has leaked and that the mouse ID is still clearly visible on the cap lid (*see* **Note 14**).
4. Centrifuge the tubes at 14,000*g* in a microcentrifuge for 10 min. Take the tubes out, being careful not to disturb the interface. Transfer the supernatant to a clean tube marked with the appropriate mouse ID, and add 500 µL of isopropanol.

5. Carry out **steps 5–12** as in **Subheading 3.2.2.1.**
6. Add 50 μL of TE and heat the samples at 65°C in a heating block for 15 min or at 4°C overnight, to dissolve the DNA. This is the stock DNA. Store at 4°C.
7. Dilute the stock DNA samples by mixing 5 μL of DNA with 45 μL of TE. Store at 4°C. One microliter of diluted DNA should contain sufficient template for a 25-μL PCR reaction.

## 4. Notes

1. Embryo transfer pipets are made by heating a glass capillary, pulling it to make it thinner, cutting the pulled end with a diamond cutter, and bending the capillary about halfway up the pulled end to a 90° angle using heat from a Bunsen burner.
2. The blastocyst donor mouse strain will depend on the strain of ES cell used. Because most ES cells in current use are derived from 129 strains, the blastocyst donor of choice is the C57BL/6 strain. This strain's embryos are compatible hosts for 129 cells. Using C57BL/6 blastocysts makes identification of chimeras easy by observing the coat color of the offspring. Chimeras with a high ES cell contribution will be mostly agouti in color, whereas those with low ES cell contribution will be mostly black. Other mouse strains can be used, but it is advisable to use strains of defined genetic background to make subsequent breeding of a pure line easier.
3. If the mice have only just arrived at the animal facility, they may not produce any plugs when they are first mated. Usually sufficient blastocysts can be obtained from 12 to 15 plugged females.
4. The choice of strain for the foster mice will usually depend on the availability of outbred strains in individual facilities. We have successfully used the CD1 strain because the females make very good mothers; others use a mixed strain of DBA/BL10. Because the foster mother does not contribute genetically to the embryos, the strain should be chosen on the basis of suitability and cost. In order to get pseudopregnant females, matings should be set up between receptive females and vasectomized males. Males can be purchased already vasectomized from most common suppliers or can be operated in-house. For a full vasectomization procedure, *see* **ref. *3***.
5. After flushing the horns, various elements can be seen, from eggs to morula, and because only the blastocyst can be injected, these must be correctly identified. There are good photographs of blastocysts in **ref. *3***, but it is always best to get someone who is experienced to show the difference.
6. It is advisable to use the anesthetic recommended by the local animal technicians, because they will have a better idea of the right dose and recovery times. We have used a mixture of Hypnorm/Hypnovel/water (1:1:2 [v/v/v]) at a dose of 150 μL/animal of average weight (25–30 g).
7. The number of pups is extremely variable and depends on the skill of the person carrying out the transfer procedure and on whether the foster mother is truly pseudopregnant. In theory, all blastocysts should give rise to pups, and because they have all been injected, all pups should be chimeric. However, in practice, the majority of embryos do not implant, and most litters are of two to four pups. In a very successful transfer, four to five pups will be born and three or four will be chimeras. It is not uncommon for pregnancies to be lost at the early stages and for mothers not to rear the young if the litters are too small (i.e., only one or two pups). In our hands, it is necessary to inject at least 100 blastocysts to guarantee the birth of male chimeras that will transmit through the germ line.
8. If the first test-cross litter has no agouti pups, it is worth crossing the chimera again; sometimes germ-line transmission will occur in subsequent litters. In practice, it is often

advisable to mate the male chimeras to several females without waiting for the first litter to be born. In a mating cage, the chimera can be placed with two females at a time, vaginal plugs can be checked every day, and any plugged mice can be transferred to separate cages. The female can then be replaced with another one. If it is not practical to check plugs every day, females can and should be rotated once a week, to maximize the number of potential pregnancies and, hence, the possibility of obtaining germ-line transmission.

9. There are recommendations set out as to how to breed KO mice in order to produce congenic and coisogenic lines ***(2)***.
10. Mice are sexed by looking at the distance between the anal and urinary orifices. The orifices are closer together in a female compared with a male. Sometimes in females mammary glands can also be distinguished.
11. The cages we use will only hold five mice, so this numbering is adequate. If cages are bigger more combinations can be added to distinguish the mice. The complete ID of a given mouse will be, e.g., "Sam N6 2M3." First comes the name of the chimera from which it was derived (Sam), followed by the generation (N6), the litter number (2), the sex (M = male), and the mouse number (3). A mouse with this ID will be found in a cage labeled "Sam N6 litter 2 males," and it will have one punch hole in its right ear. It is essential to keep a record of all matings, births, and weaning. This can be kept as a spreadsheet and, as a safeguard, in hard copy. Each record should contain any information relevant to a given mouse. The headings should include mouse ID, father's ID, mother's ID, sex, date of birth, coat color, strain (e.g., chimeraXC57BL/6), genotype, and other comments such as use of the animal in a particular experiment.
12. We have used PCR primers for the *HPRT* minigene to distinguish between WT and heterozygous mice. Mice that have been derived from the mutated ES cells and thus have a deletion of a region of Chromosome 1 will carry along with the deletion an *HPRT* transgene that is different from the endogenous *Hprt* gene in that it is human in origin and has only one intron. PCR primers can be designed for any of exons 3–9 that will amplify only from the introduced transgene. WT mice will not carry the *HPRT* transgene. Later, to distinguish between heterozygous and homozygous mice, we used primers to amplify the genes within the deletion (*Fmo1, Fmo2,* or *Fmo4*). A mouse that has the *HPRT* minigene but not, e.g., *Fmo1* must be homozygous for the deletion.
13. Tails must be incubated until adequate cell lysis has occurred. We usually leave tails lysing overnight (about 16 h), but they can be left longer.
14. Always use a permanent marker pen. Usually black and blue ink are better; red and green ink tend to be erased easily with phenol and leave no trace of the writing.

## Acknowledgment

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# 37

# Use of Reporter Genes to Measure Xenobiotic-Mediated Activation of *CYP* Gene Transcription

**Nick Plant**

**Summary**

Transcriptional activation of *CYP* gene expression by xenobiotics may have fundamental effects on body physiology. It may result in the altered pharmacokinetics of other chemicals in the body, both xenobiotic and endogenous substrates, potentially altering their effects. This may often result in no observable clinical effect, but in a significant number of cases these interactions lead to altered physiology or failure of a therapeutic drug. It is therefore important to be able to screen novel chemical entities for their ability to activate *CYP* gene expression. In addition, through mechanistic studies of how such transcriptional activation occurs, the ability to predict and avoid such potential interactions is improved. Reporter gene assays provide a simple, high-throughput methodology for examining the transcriptional activation of *CYP* gene expression by xenobiotics. They are suitable for use in screening as well as mechanistic studies and are of use in both the drug discovery/development and research arenas.

**Key Words:** Transcription; *CYP*; reporter gene; nuclear receptor; Hep G2; Huh7; drug–drug interaction; transfection.

## 1. Introduction

Many cytochromes P450 (CYPs) have been shown to be regulated by xenobiotics at the transcriptional level; increased amounts of xenobiotic result in an increase in the rate of transcription of the *CYP* gene whose product is responsible for metabolizing the xenobiotic *(1)*. This responsive mechanism therefore leads to more efficient removal of the xenobiotic. However, it may also alter the pharmacokinetics of any coadministered xenobiotics, changing their effect and, in the case of therapeutic agents, potentially the clinical outcome *(2)*. To understand and, ultimately, predict such drug–drug interactions, it is thus important to understand the molecular mechanisms underlying *CYP* transcriptional activation by xenobiotics; reporter gene assays provide an excellent tool for doing so.

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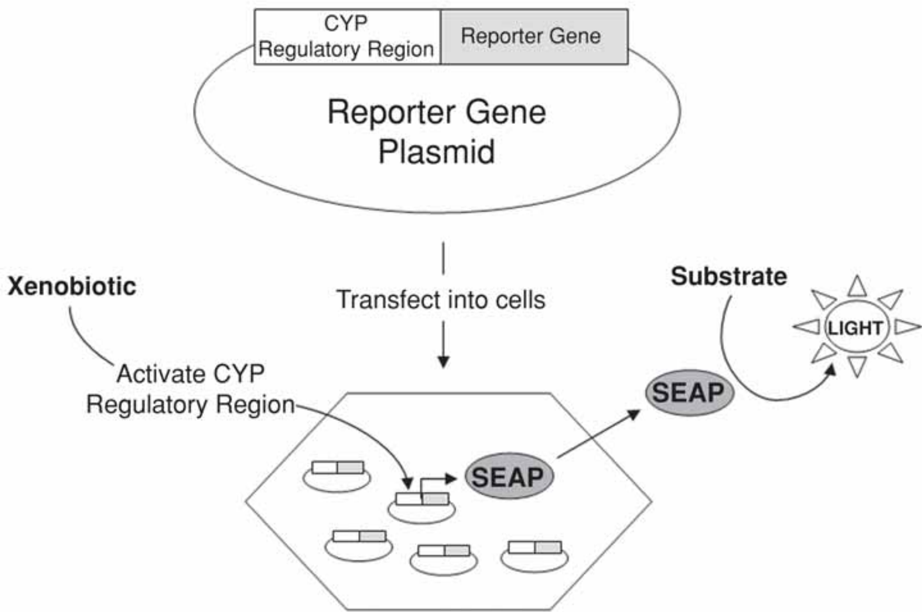


Fig. 1. Overview of the reporter gene methodology for measurement of xenobiotic-mediated activation of *CYP* gene transcription.

This chapter provides an overview of reporter gene assays for the measurement of *CYP* gene transcriptional activation. Many different methods exist for undertaking reporter gene assays, and it is hoped that the information presented in this chapter will help researchers select the most appropriate system for their particular needs.

### 1.1. Why Undertake Reporter Gene Assays?

A valid question is, Why use reporter gene assays at all? To answer this, one must first define what is meant by a reporter gene. **Figure 1** shows a simple illustration of a reporter gene plasmid and its potential utility in detecting transcriptional activation by xenobiotics. As shown, reporter gene assays function by inserting a fragment of the regulatory region for the gene of interest (e.g., CYP3A4) upstream of the coding region for the reporter protein. Activation of the regulatory regions, which would produce an increase in the amount of the corresponding CYP protein in vivo, thus causes an increase in the expression of the reporter protein, which is then assayed to provide a measure of transcriptional activation.

The major advantage of a reporter gene is exactly what the name suggests; that the expression of an easily measured surrogate marker is used rather than the native gene product. Because the technologies for detection of these reporter proteins are well defined, and because these surrogate markers are not normally expressed within the cell, it is thus possible to design a specific assay for the transcriptional activation of

any desired gene. This was an obvious advantage when the ability to detect individual CYP enzymes was poor. However, it could be argued that with the development of highly specific probe sets for quantitative reverse transcriptase polymerase chain reaction (RT-PCR) even very similar CYPs (e.g., CYP3A4 vs CYP3A5) can now be distinguished. Hence, those with such technology could argue that it has obviated the need for reporter assays. However, the reporter assay has several advantages over measurement of mRNA changes elicited in cells on exposure to xenobiotics. These advantages are as follows:

1. *Improved control of experiments:* If the correct reporter is chosen, then several readings can be taken from a single well, thus allowing close control of experiments.
2. *Mechanistic analysis:* Because only a part of the regulatory region is used, it is possible to carry out mechanistic experiments on the role of differing sections of the regulatory regions, through the use of deletion or site-directed mutants.
3. *Utility of human cell lines:* Many studies on transcriptional activation are undertaken in human cell lines because these lend themselves to such high-throughput analysis. However, because chromatin conformation may be dramatically different in a cell line compared to in vivo, they do not necessarily represent a good model for quantitative RT-PCR measurements of transcript levels produced from genomic copies of a gene. Activation of nonchromosomal reporter genes is not affected by Chromatin status, removing this potentially confounding issue.

It can thus be seen that reporter genes fill a specific niche within the battery of technologies available to study CYP function; they provide the ability to undertake mechanistic examinations of the activation of *CYP* gene expression by xenobiotics, as well as provide a format for high-throughput examination of such activity.

### 1.2. Overview of Methodology

**Figure 1** provides a simple overview of reporter gene technology. A reporter plasmid is first constructed from the regulatory region of the *CYP* gene of interest linked to the coding region of an easily measurable reporter gene. This is then transfected into an appropriate cell line and the effect of exposure to xenobiotic is examined. However, several key questions need to be answered before an experiment can be undertaken, to ensure that the most accurate information is gained. These are: Which/how much regulatory region should be used (*see* **Subheading 1.2.1.**)? Which reporter should be used (*see* **Subheading 1.2.2.**)? Which cell line should be used (*see* **Subheading 1.2.3.**)?

#### 1.2.1. Choice of Regulatory Region

Perhaps the first question facing the researcher is, what regulatory regions should be used and, just as important, how much of them should be included in the reporter construct? In many ways, this question is akin to how long is a piece of string? Because there is no definitive answer, but some general guidelines can be given. The potential regulatory regions that could be included in a reporter construct are threefold.

1. *Single response element:* Using a single response element, often as a concatemer, allows investigation of the role of a single transcription factor in mediating the xenobiotic

activation of transcription to be studied. Although, strictly speaking, this is not a reporter assay for an individual CYP, it can be used to examine the role of the major nuclear receptors involved in *CYP* gene expression. For example, activation of a reporter gene containing a triplicate repeat of the PXR response element is often used as a surrogate marker for *CYP3A* gene activation ***(3)***.

2. *Proximal promoter region:* Because a single response element will not provide definitive data on activation of an individual *CYP* gene, the logical extension is to use a region of the proximal promoter for the CYP of interest. This will then include the response element(s) for major nuclear receptors (e.g., PXR, in the case of CYP3A4), but also response elements for other transcription factors that may modulate the xenobiotic-mediated gene activation (e.g., C/EBPα and HNF3, in the case of CYP3A4) ***(4)***. Evidence tends to suggest that many of the important response elements controlling expression of a gene are within a few hundred base pairs of the transcription start site. Hence, a reporter construct containing 1000 bp of proximal promoter will provide good coverage of these sites ***(5)***. It should be noted that there are exceptions to the rule, with some genes having promoter regions that extend several thousand base pairs from the transcription start site. It is thus important to review each *CYP* promoter on a case-by-case basis and decide the most appropriate length of promoter to include in a promoter-reporter gene construct. It is probably better to err on the side of caution with a large region of DNA and then reduce this as experiments show the vital regions; for example, we have moved from using 1000 bp of *CYP3A4* proximal promoter ***(5)*** to only 300 bp ***(4)***.
3. *Enhancer region:* Expression of a gene may be controlled not only by the proximal promoter but also by enhancer elements. Enhancer elements serve both to increase the degree of expression and to refine it for any specific gene. The use of a heterologous enhancer, such as the enhancer cytomegalovirus or SV40t, can be used for the first function—increasing signals to a poin which they can be easily measured. However, the second function can only be carried out by the native enhancer element. The problem is that for many CYPs, and indeed many genes in general, enhancer elements have not been fully defined and, therefore, cannot be included in a reporter gene construct. Use of a heterologous enhancer may be necessary in these circumstances to provide a detectable signal, but, if possible, the identified cognate enhancer elements should be included in any reporter construct (e.g., the XREM for CYP3A4) ***(4,6)***.

### 1.2.2. Choice of Reporter

There exist several different reporter genes, all of which may be capable of acting as a simply detected surrogate marker of transcriptional activation. Which, then, is the most appropriate to choose? In essence, the multitude of possible reporter genes may be subdivided into three categories, depending on the type of output that they can produce:

1. *Colorimetric:* The original, and still widely used, reporter gene is β-galactosidase ***(7)***. Production of this enzyme was classically detected by the metabolism of 5-bromo-3-chloro-3-indoyl-β-D-galactoside, producing an insoluble blue product, which can then be detected by absorbance spectroscopy. Although still of some utility, colorimetric reporter assays have largely been replaced by the second and third options, which show distinct advantages.
2. *Luminescent:* An alternative end point for enzyme-based reporter genes is a luminescent output. Many enzymes, including β-galactosidase ***(8)***, alkaline phosphatase ***(9)***, and luciferase ***(10)***, may also catalyze enzyme reactions producing luminescent products.

Luminescence detection has the advantage of increased sensitivity, and the addition of secretory tags to the enzyme produces a product that can be assayed directly in culture medium ***(11)***. Negation of the requirement for cell lysis before measurement allows repeat readings from a single well, thus increasing the ability to control experiments.

3. *Fluorescent:* The final type of reporter gene produces a protein that, rather than catalyzing an enzyme reaction giving an indirect measure of the level of reporter protein, is itself directly measurable. Fluorescent proteins emit light on excitation and, thus, produce a simple end point that requires no further processing to produce a reading ***(12)***. As with secretory luminescent systems, fluorescent proteins do not require cell lysis for measurement and, hence, multiple readings can be taken from a single well. In addition, because fluorescent reporters require no chemical processing to detect, they are both faster and cheaper. Finally, several different colors exist for fluorescent proteins, raising the possibility of using multiple reporters in a single cell. This may be of particular interest in the CYP-reporter field, because it would allow the study of how one xenobiotic may alter the expression of several CYPs simultaneously. The major disadvantage of fluorescent systems is that they have a lower sensitivity than enzyme-based systems, because the signal does not go through an amplification stage.

In my laboratory, we use the latter two end points, luminescent and fluorescent. Where the expected output signal is strong, fluorescent-based end points provide a cheap, flexible system. However, for the majority of our CYP-based reporter assays, we utilize secreted alkaline phosphatase (SEAP) as a luminescent end point. Many CYPs are expressed only at low levels under basal conditions, with the expression increasing manyfold on addition of xenobiotic. However, to allow accurate determination of a fold induction for *CYP* reporter gene expression, it is necessary to measure the basal level of expression; in general, we find this at or below the level of detection of fluorescent systems. Using a chemiluminescent system, however, increases sensitivity and allows the basal level to be easily measured with a high degree of confidence, providing a much more robust reporter gene system.

### *1.2.3. Choice of Cell Line*

The choice of cell line to carry out reporter gene assays is an often-overlooked parameter, but one that can be central to producing mechanistically valid results. Because the majority of CYPs are expressed in the liver, a sensible starting point is to choose a liver cell line. However, a simple search of the American Type Culture Collection and European Collection of Cell Cultures repositories reveals more than 10 different human liver cell lines; which, then, is suitable for reporter gene studies? At the simplest level, the cell line should act as a bag of transcription factors, containing all the relevant proteins for the acquisition and transfer of xenobiotic signal to the reporter gene. In addition, the cell should ideally have a full complement of membrane-bound drug transporters, thus allowing access of the xenobiotics to the cell interior. Finally, not only must all these proteins be present, but they must ideally be expressed in the correct amounts to mimic the expression in vivo; only if this is achieved can a truly accurate model be made. The transcriptomic and/or proteomic analysis of many cell lines is now being undertaken and, thus, these parameters can be checked. Thus far the evidence in the literature suggests that Huh7 ***(13)*** and HepG2

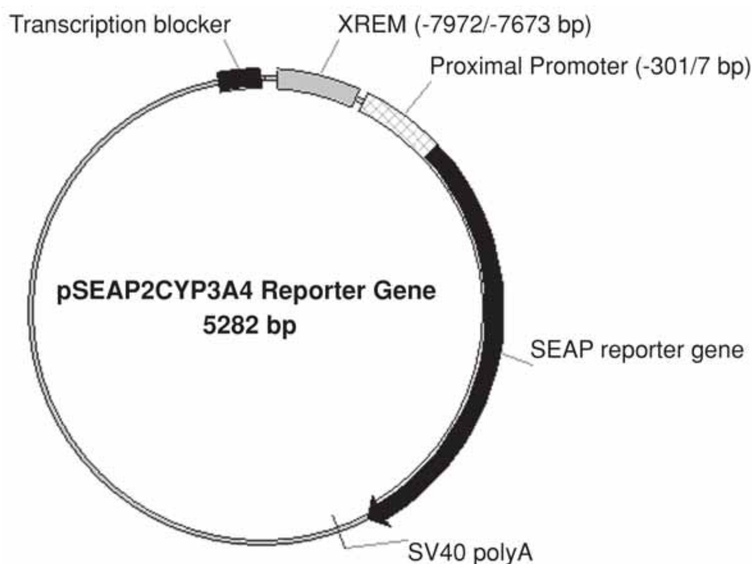


Fig. 2. Schematic representation of the CYP3A4 reporter gene construct.

***(14)*** cell lines represent the in vivo liver reasonably well. However, it should be noted that neither of these cell lines is ideal, and they do not express the full complement of transcription factors/transporters ***(15)***. One way around this is to cotransfect with an expression plasmid for the key transcription factor involved in the xenobiotic-mediated activation of the CYP under study. Hence, we cotransfect an expression plasmid for human PXR with all *CYP3A* reporter gene studies, increasing the relatively low expression levels of PXR in both Huh7 and HepG2 cells ***(5,15)***. Note, however, that this approach is not without its problems: because transcription factors work together in an interaction network, increasing the amount of a single factor by several orders of magnitude may skew results toward this pathway. Therefore, regardless of the adopted system it is vital that the reporter assay be fully validated before experimental use (*see* **Note 1**).

## 2. Materials

### 2.1. Cell Culture

1. Cell lines: The human hepatoma cell lines HepG2 (ECCAC No. 85011430) and Huh7 (a gift from Dr, Steve Hood, GlaxoSmithKline UK) are routinely used for *CYP* reporter gene assays.
2. Plasmids: Reporter constructs are routinely made in pSEAP2basic (BD Biosciences, Cowley, UK) and pEGFP-1 (BD Biosciences) vectors for luminescent and fluorescent end points, respectively. The CYP3A4 reporter construct described in **Fig. 2** contains the *CYP3A4* enhancer module (XREM;–7972 to –7673 bp) and 308 bp of proximal promoter (–301 to +7 bp), cloned directly upstream of the SEAP-coding region of pSEAP2basic (**Fig. 2**).

3. Medium: Cells are cultured in either minimum essential medium (MEM) (HepG2) or Dulbecco's modified Eagle's medium (DMEM) (Huh7), both supplemented with 10% (v/v) fetal calf serum, 1% (v/v) nonessential amino acids, 2 m*M* L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. All medium components were purchased from Invitrogen (Paisley, UK).
4. Plasticware: 75-$cm^2$ vented culture flasks and 96-well culture plates were purchased from Nunc A/S (Roskilde, Denmark).

### *2.2. Transfection*

1. DNA: All DNA to be used in transfections is prepared using an Endo-free maxiprep kit (Qiagen, Crawley, UK), because the presence of endotoxins has been shown to reduce transfection efficiency in some cell lines ***(16)***.
2. Transfection agent: Transfections were carried out using FuGENE6 (Roche, Lewes, UK).

### *2.3. Quantification of Reporter*

1. SEAP assay: Chemiluminescent detection of SEAP activity was undertaken using an Aurora AP Chemiluminescent Assay (MP Biomedicals, Costa Mesa, CA).
2. Materials: Microplates used for chemiluminescent detection were Optiplate-96 (Perkin Elmer, Zaventem, Belgium).

## 3. Methods

### *3.1. Cell Culture*

1. Thaw one cryopreserved vial of human hepatoma cells in a water bath at 37°C. Each vial contains approx $10^6$ cells in 1 mL of 9% fetal bovine serum, 9% dimethyl sulfoxide.
2. Transfer the cells into a 75-$cm^2$ flask with a vented lid, and add 15 mL of supplemented medium. HepG2 (ECCAC no. 85011430) and Huh7 (GlaxoSmithKline UK) human hepatoma cells require supplemented MEM and DMEM, respectively. Incubate the cells in 5% $CO_2$ in air at 37°C.
3. Every 3–5 d, when the cells have approached 90% confluence in the flasks, subculture at a ratio of 1:4.
4. Culture the cells for 1 wk following retrieval from liquid nitrogen before starting experiments.
5. Discard the cells after 3 wk of culture to ensure phenotypic consistency.

### *3.2. Transfection*

#### *3.2.1. Seeding of Cells*

1. Before transfection, culture cells to approx 90% confluence in 75-$cm^2$ flasks.
2. Harvest the cells by trypsinization, and collect by centrifugation (250*g*, 5 min).
3. Remove the supernatant by aspiration and wash the pellet in 5 mL of phosphate-buffered saline.
4. Resuspend the washed cell pellet in 5 mL of supplemented medium (*see* **Subheading 2.1., item 3**).
5. Determine cell density using a hemocytometer. During counting, store the remainder of the cells on ice.
6. Adjust the cell suspension to a concentration of $10^5$ cells/mL.
7. Add 120 μL of cell suspension into each well of a 96-well plate (120 μL/well is equivalent to 12,000 cells/well).

8. Return the cells to the incubator for 24 h to allow attachment (*see* **Note 2**). Be sure to place the 96-well plate in a humidified chamber within the incubator to prevent evaporation. The number of wells required for each experiment is detailed in **Note 3**.

#### 3.2.2. Transfection of DNA

1. Prepare transfection mix: serum-free supplemented medium (to produce a final volume of 100 µL), 15 µL of FuGENE6, and 5 µg of plasmid DNA. If more than one plasmid is used, then the quantity of each plasmid is reduced so that the total amount of plasmid added equals 5 µg (*see* **Note 4**).
2. Incubate the transfection mix at room temperature for 45 min to allow the formation of micelles.
3. After incubation, add 9.5 mL of serum-free supplemented medium (*see* **Note 5**).
4. Remove the medium from the 96-well plate containing seeded cells by inverting and shaking the plate.
5. Add the transfection mixture to the seeded 96-well plate (100 µL/well).
6. Incubate the plates at 37°C for 24 h in a humidified chamber.

#### 3.2.3. Cell Dosing

After the transfection period, cells are ready for exposure to xenobiotic (*see* **Note 6**).

1. Remove 100 µL of medium and store at –20°C for later determination of the predose SEAP activity.
2. Remove residual medium by inverting and shaking the plates.
3. Add 120 µL of fresh medium containing either xenobiotic or vehicle alone.
4. Incubate the plates at 37°C for 48 h in a humidified chamber.
5. Remove 100 µL of medium and store at –20°C for later determination of the postdose SEAP activity (*see* **Note 7**).
6. Discard the plates.

### 3.3. Quantification of Reporter

The amounts of alkaline phosphatase secreted into the cell-culture medium are assessed using the Aurora AP chemiluminescent Assay with a modification to the manufacturer's protocol.

1. Transfer 10 µL of medium from each well to a 96-well optiplate (polystyrene microplates).
2. Add 18 µL of 1X dilution buffer.
3. Incubate at 65°C for 30 min to inactivate any endogenous alkaline phosphatase activity.
4. Cool on ice to room temperature.
5. Add 24 µL of assay buffer and incubate for 5 min at room temperature.
6. Add 24 µL of reaction buffer (containing luminescent substrate) and incubate for 20 min at room temperature.
7. Read the luminescent output on a Lumicount plate reader (Packard). Use automatic detection of optimal gain and photomultiplier settings.

### 3.4. Data Analysis

Data analysis is carried out to eliminate nonspecific effects and calculate the fold induction for each xenobiotic ***(17)***. If a reporter activity is measured for a series of concentrations of a single xenobiotic, the values are then plotted and the maximal

predicted induction ($I_{max}$) and the concentration of xenobiotic required to reach half-maximal induction ($EC_{50}$) are calculated ***(5)*** (*see* **Note 8**).

1. If necessary, adjust pre- and postexposure reading to account for any autoscaling function of the plate reader (*see* **Note 9**).
2. Subtract the preexposure value from the postexposure value for each well.
3. Subtract the value for the blank control plasmid from the respective *CYP* promoter reporter gene plasmid value. Do this for each concentration of xenobiotic used and for vehicle-treated cells. This calculates the effect of xenobiotic/vehicle exposure on the *CYP* promoter–specific activation of the reporter gene construct.
4. Divide the value for each xenobiotic by the value for the vehicle-specific effect. This provides the fold induction for the xenobiotic on the *CYP* regulatory elements under study.

The data analysis is summarized in the following equation:

$$\frac{\left[(\overline{X_{post} - X_{pre}})_{CYP} - (\overline{X_{post} - X_{pre}})_{blank}\right]_{Xenobiotic} \quad ①}{\left[(\overline{X_{post} - X_{pre}})_{CYP} - (\overline{X_{post} - X_{pre}})_{blank}\right]_{Vehicle} \quad ②}$$

Statistical analysis can then be carried out.

1. Calculate the variance of each data point set: For example, the variance of the six repeats of xenobiotic exposure on one plasmid.
2. Combine the variance for each step in the data analysis. This gives the total variance of the data used to derive each fold induction.
3. Insert these variance values, along with the fold induction, into the Students t-test. Thus,

$$\frac{① - ②}{\left[VAR^{xeno}_{CYP} + VAR^{vehicle}_{CYP} + VAR^{xeno}_{blank} + VAR^{vehicle}_{blank}\right]^{1/2}}$$

If a concentration range of xenobiotic is used, then further characteristics of the induction profile can be gained.

1. Input data into a suitable graph-drawing package, such as GraphPad Prism v4.
2. Fit a curve to the data using the hyperbola equation $Y = I_{max} \times X/(EC_{50} + X)$.
3. Accept the fitted curve if $r^2 > 0.9$. If lower than this number, repeat the experiment with an adjusted concentration range to allow accurate curve fitting.
4. $I_{max}$ is the predicted/observed maximal induction for the test chemical. $EC_{50}$ is the concentration of test chemical required to reach half-maximal induction. Divide the $I_{max}$ value by the $EC_{50}$ to calculate the overall inductive ability (*IA*) of a test chemical. This allows a more systematic ranking of the ability of xenobiotics to activate *CYP* gene expression.

## 4. Notes

1. It is important that any assay be fully validated in-house before its full use. Reliance on the validation of others is not optimal, because lab-to-lab variations, both in the way

techniques are carried out and in the properties of cell line being used, may make such comparisons invalid. Probably the simplest validation system is to use a "training set" of 10 or more xenobiotics whose characteristics in vivo are well known. All xenobiotics from the training set would be correctly ranked as inducers/noninducers in a perfect system; however, very often this is not the case. Through study of incorrectly ranked compounds, it is possible to gain information on the type of chemicals that are incorrectly called and, thus, provide the caveats for the reporter system.

An alternative approach is the multivariate analysis of transcriptome/proteome profiles of cell lines to identify similarities/differences between them and their relationship to the situation in vivo. Although these data will not currently allow prediction of how a reporter system will respond to any given xenobiotic, they will allow predictions on the utility of a cell line for use in *CYP* reporter gene assays.

2. It is clear that cell lines grow and behave differently at different plating densities. It is therefore important to constantly observe the cells to check their confluence and base timings rather than sticking rigidly to the provided times. In particular, after seeding into 96-well plates, cells are allowed to attach for 24 h before transfection. Cells must be checked the morning of transfection, and if confluence is not >50%, then the cells should be allowed to grow further. Conversely, if cell coverage is >80%, the cells should be reseeded because they run the risk of overgrowing during the experimental period.
3. We use six wells for each data point, although this may be reduced to four without too significant a decrease in the discriminative power of the data analysis. Data points are collected for the effect of each xenobiotic concentration or vehicle alone on the *CYP* reporter gene plasmid construct and on the blank plasmid (e.g., one containing no *CYP* regulatory sequences). In addition, each plate contains a positive control (e.g., 10 m*M* rifampicin for a *CYP3A4* reporter gene); this provides an internal control for interexperimental variation and allows accurate comparison between plates.
4. There are two key issues in preparing the transfection reagent. First, all components must be added in the order listed. Failure to do so may result in incorrect formation of micelles and reduced transfection efficiency. Second, the FuGENE reagent must be mixed into the medium and not allowed to come into contact with the sides of the vessel. Owing to its highly lipophilic nature, it will readily adsorb onto plastic surfaces and incorrect introduction may lead to significant loss of transfection efficiency.
5. The final volume (9.5 mL) of transfection reagent is sufficient for a single 96-well plate. Volumes may be scaled appropriately, for larger/smaller numbers of wells, with no apparent loss of transfection efficiency.
6. We have tested various time periods for the exposure of the cells to xenobiotics and have settled on 48 h for several reasons. First, there is often an appreciable increase in signal between 24 and 48 h of exposure, increasing the dynamic range of the experiment. Although this does not change the rank order of xenobiotic induction in any way, it does increase separation and, thus, makes interpretation of data easier. Second, increasing exposure to 72 h does not dramatically increase the dynamic range. Third, a 48-h exposure period ensures that the cells do not overgrow in the 96-well plate, which could potentially skew results. Finally, using a 48-h exposure means that the entire reporter gene assay can be easily fitted into a working week.
7. It is important to inspect the cells visually before removing the postexposure medium sample. Any sign that the cells have had their physiology altered by xenobiotic exposure should be noted, including increased growth rates and, more normally, toxicity. If either situation is observed, then the experimental protocol may have to be altered to correct for

this problem. Decreasing initial plating density, exposure time, or xenobiotic concentration may all be applicable to prevent/reduce the effect to an acceptable level.

Even if chemiluminescent detection is to be carried out immediately, it is worthwhile to subject the postexposure medium samples to a freeze-thaw cycle, because this has been done with the preexposure samples. Although SEAP appears to be relatively stable, this simple step further reduces variation in the system.

8. Through the use of a nondestructive end point (e.g., SEAP, fluorescence), it is possible to take a reading from the same well before and after exposure to xenobiotic. The preexposure reading acts as an internal control, allowing normalization for variations in transfection efficiency. Although this can be achieved by cotransfection with a second, noninducible reporter gene, we feel that this system has several advantages. First, there is no need to carry out a separate assay for the "transfection control" reporter plasmid because this can be incorporated into the assay for the effect of xenobiotic. Second, measurement of the *CYP* reporter gene itself eliminates any variation between it and a cotransfected "transfection control" reporter plasmid, which might skew the data. Finally, using the *CYP* reporter gene also controls for other sources of variation, such as activation of the reporter gene by endogenous ligands, which may vary among wells but be specific to the reporter gene construct.
9. It is important to note that pre- and postexposure readings must be made on the same scale for the analysis to function. Many plate readers use an autoscaling function to maximize the signal produced. Because the preexposure readings will almost certainly be lower than the postexposure readings, they may well be scaled differently. The raw data must be adjusted for any such scaling factor (depending on the plate reader/software used) before further analysis can be undertaken.

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