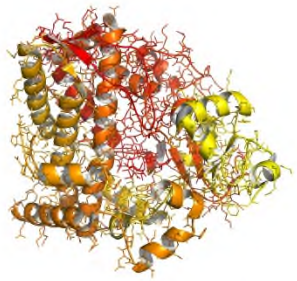

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RECONSTITUTION OF PURIFIED RECOMBINANT HUMAN P450 ENZYMES

A protocol is given for reconstituting recombinant (**r**) human P450 enzymes purified from Sf9 insect cells or E.*coli* with human liver P450 reductase (Fp) and cytochrome b_5 (b_5). The phospholipid used is dilauroylphosphatidylcholine (DLPC), since reconstitution with this lipid appears to gives optimal catalytic activity with all human P450s examined (except in the case of CYP3A4; see below).

In the experiment shown below, two recombinant human P450s (**r**CYP2C9 and rCYP2C19) are being tested for their ability to metabolize a given substrate. Each reconstituted system is prepared in a quantity sufficient to assess metabolism by both enzymes ± b_5 in triplicate. The amounts of **r**CYP2C9 and rCYP2C19 included in the reconstituted system are such that product formation rates are proportional to the amount of P450 added. The P450:Fp, P450:b_5, and P450:DLPC ratios have been optimized in preliminary studies.

SET-UP FOR RECONSTITUTED SYSTEMS (RCS)

Enzyme	Concentration
Human **r**2C9	5.0 nmol/ml
Human **r**2C19	5.0 nmol/ml
Human Fp	15.7 nmol (55 kU)/ml
Human b_5	25.8 nmol/ml
DLPC (Serdary)	1 mg/ml in H_2O (see ☆ below)

ORDER OF ADDITION TO 10 x 75 mm GLASS TUBES KEPT @ ROOM TEMP:
DLPC ➔ Fp ➔ P450 ➔ b_5 - Vortex After Each Addition

	DLPC	Fp	b_5	P450	λ/assay
RCS-**A**	19 λ	41λ	10 λ	--	22 (n=3)
RCS-**B**	19 λ	41 λ	--	13λ **rCYP2C9**	23 (n=3)
RCS-**C**	19 λ	41 λ	10 λ	13λ **rCYP2C9**	26 (n=3)
RCS-**D**	19 λ	41 λ	--	13λ **rCYP2C19**	23 (n=3)
RCS-**E**	19 λ	41 λ	10 λ	13λ **rCYP2C19**	26 (n=3)

RCS-A contains **NO P450**, 200 pmol (650 units) Fp, 6 µg DLPC and 80 pmol b_5.
RCS-B ➔ RCS-E contain 20 pmol P450 enzyme, 200 pmol Fp, 6 µg DLPC **±** 80 pmol b_5.

NOTE #1: The amounts of DLPC, Fp, and P450 enzyme used to form each reconstituted system are slightly greater than that required for triplicate assay tubes. For example, while only 18 λ (18 µg) of DLPC, 38λ (600 pmol) Fp and 12 λ (60 pmol) rCYP2C9 are needed to run 3 tubes with RCS-B, 19λ DLPC, 41λ Fp and 13λ rCYP2C9 are actually added so that 23λ of the mix can be readily retrieved for each of the 3 assay tubes.

Once All The Above Components Are Added:

1. Incubate tubes containing RCS @ 37°C for 5 min, then place back onto ice.

2. Add the appropriate amount of each RCS to the tubes in which the metabolism reactions will be performed. These tubes should at least contain assay buffer (e.g, 50 mM HEPES).

3. Add the remainder of the reaction components.

4. Start reactions with NADPH.

☆Prepared fresh by adding 100λ of DLPC stock solution [10 mg/ml in $CHCl_3$] to a 13 x 100 mm glass tube, evaporating off **ALL** the $CHCl_3$ with a gentle stream of nitrogen, adding 1.0 ml of H_2O, and resuspending using 4 x 15 sec bursts of a probe-type sonicator set @ 100 watts (**NOTE:** cool solution for 30 seconds on ice between bursts).

NOTE #2: The phospholipid used for rCYP2C9 and rCYP2C19 reconstitution, like that utilized with nearly all other P450 enzymes, is DLPC. The only human P450 enzyme that exhibits a different phospholipid requirement is rCYP3A4, which must be reconstituted with dioleolyphosphatidylcholine (DOPC) to obtain enzymatic activity.