RECO® System CYP2D6 Simplified Reconstitution Assay

Product # P2775

1.0 INTRODUCTION

PanVera provides a product that greatly simplifies the steps involved in reconstituting purified cytochrome P450 isozymes and their associated proteins. The amount of reconstitution mix and the volume used for an assay will depend on several factors:

- sensitivity of the analytical assay for the metabolites and catalysis products
- kinetic parameters of enzyme catalysis for a particular substrate and P450, if known
- incubation period of the assay
- solubility of the test compound or substrate
- preference of individual research groups

Details of general assays[1,2,3] and reconstitution methods[4,5,6] have been published. Most details of specific substrate assays for individual isozymes of cytochrome P450 have also already been published:

Isozyme	Substrate
CYP1A1	Ethoxyresorufin[7]
CYP1A2	Methoxyresorufin[7]
CYP3A4	Nifedipine, Testosterone, Midazolam, Erythromycin[4,5,8,9]
CYP2C9	Diclofenac[10,11]
CYP2C19	Mephenytoin[11,12,13]
CYP2D6	Bufuralol, Debrisoquine, Dextromethorphan[11,14]
CYP2E1	N-nitrosodimethylamine, Chlorzoxazone[15,16]

Individual laboratories have modified the assays to suit their personal preferences. When using a substrate whose kinetic parameters are not known, the following conditions may serve as a useful starting point:

Reaction Volume	0.1-0.5 mL
CYP2D6 (5X Enzyme Mix)	10-50 pmol
Substrate Concentration	100-500 µM
Incubation Time	5-10 min

A pre-incubation period of 2-3 minutes is often performed before the reactions are initiated by the addition of the final compound (substrate, or either NADPH or a regenerating system, if preferred). The linearity of the assay and kinetic parameters should be determined by standard methods. Reactions are stopped by adding either methanol, acetonitrile, or acid (final concentration of ≥20%) to the incubation mixes and then placing them on ice. If an internal standard is to be used for the analytical assay, it can often be included in the stop mixture. In some cases, extracting the metabolites from the reaction mix is useful, as it enhances detection by concentrating the metabolites and removing water-soluble compounds that interfere with the analysis procedure. Typically, 1.5-5 volumes of methylene chloride or ethyl acetate can be used to extract the metabolites, although other salt additives that affect the pH and/or enhance the extraction procedure may be necessary. In cases where extraction can be avoided, the reaction is stopped (as above) and the mixture is centrifuged for 10 minutes at 4°C in order to pellet the protein. The supernatant can then be used directly for analysis.

2.0 DESCRIPTION

2.1 Materials Supplied

CYP2D6 RECO® System Enzyme and Buffer Mixes, Product No. P2775, 750 pmol

- 5X Enzyme Mix**:** Part No. P2776: 0.5 µM CYP2D6, 1 µM NADPH-P450 reductase, 0.5 µM cytochrome b_5, 0.5 µg/µL CHAPS, 0.1 µg/µL liposomes [1,2-dilauroyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-sn-glycero-3-phosphocholine, 1,2-dilauroyl-sn-glycero-3-phosphoserine (1:1:1)], 3 mM reduced glutathione, and 50 mM HEPES/KOH (pH 7.4)
- 5X Buffer Mix**:** Part No. P2777**:** 200 mM HEPES/KOH (pH 7.4), 12 mM reduced glutathione, and 150 mM $MgCl_2$

2.2 Materials Required but Not Supplied

- Stock solution of 100 mM NADPH or a regenerating system (made up in water just before starting the reactions)
- Stock solution of test drug/substrate[a], Example: 5 mM bufuralol in water
- Acetonitrile (Stop Solution)
- 10X standards, Example: 1-hydroxybufuralol in water: 0, 2.5, 5, 10, 20, 40 µM

2.3 Safety Precautions

Normal precautions exercised in handling laboratory reagents should be followed. The reagents supplied are not considered hazardous according to 29 CFR 1910.1200. The chemical, physical, and toxicological properties of these products may have not, as of yet, been thoroughly investigated. We recommend the use of gloves, lab coats and eye protection when working with any chemical reagents.

3.0 PROCEDURE

These assays are specific for the measurement of CYP2D6. The assays have been modified from the cited methods and are performed in a single microcentrifuge tube. For the RECO® System, use 10 pmol of CYP2D6 in a 100 µL reaction volume.

For a 100 µL reaction:

1. Prepare NADPH and test drug/substrate stock solutions.
2. Thaw enzyme and buffer mixes rapidly and place on ice.
3. Working on ice, combine the following in a microcentrifuge tube:

5X enzyme[b]	20 µL
5X buffer mix	20 µL
water	54 µL
substrate	4 µL
total volume	**98 µL**

Mix by gently flicking the microcentrifuge tube.

4. Pre-incubate the tube at 37°C for 3 minutes.
5. Start the reaction by adding 2 µL of the stock NADPH solution.
6. Stop the reaction after the desired incubation period by adding 25 µL of stop solution.
7. Centrifuge for ten minutes to pellet the protein before analysis.
8. Analyze by HPLC using a 4.6 mm x 70 mm C18 column using the following parameters:

Flow rate:	1.2 mL per minute
Excitation wavelength:	252 nm
Emission wavelength:	302 nm
Solvent A:	30% acetonitrile containing 1 mM perchloric acid
Solvent B:	methanol
Initial conditions:	30% Solvent B; after one minute, start gradient to 100% Solvent B over 6 minutes

An example of an HPLC chromatogram can be seen in Figure 1.

a To prevent inhibition of the reaction, the final methanol concentration should not exceed 1%.
b For N-demethylation reactions, it may be necessary to use twice as much enzyme premix depending on the sensitivity of the analytical assay.

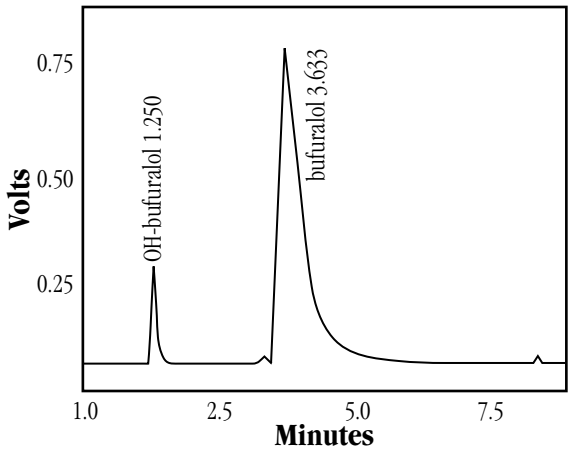

Figure 1. Sample plot of HPLC data.

4.0 REFERENCES

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