RECO® System CYP1A2 Simplified Reconstitution Assay
Product # P2304

1.0 INTRODUCTION

PanVera's RECO® Systems greatly simplify 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	0.5-1.0 mL
CYP3A4 (5X Enzyme Mix)	50-100 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

CYP1A2 RECO® System Enzyme and Buffer Mixes, Part #P2304, 750 pmol

- Enzyme Mix**:** Part #P2374: 0.5 µM CYP1A2, 0.2 µM NADPH P450 reductase, 0.5 µg/µL CHAPS, 0.1 µg/µL liposomes [dilauroyl phosphotidylcholine, dioleoyl phosphotidylcholine, dilauroyl phosphotidylserine (1:1:1)], 3 mM reduced glutathione, and 50 mM HEPES/KOH (pH 7.4).
- Buffer Mix**:** Part #P2375**:** 1 M potassium/sodium phosphate (pH 7.4).

2.2 Materials Required but Not Supplied

- Stock solution of 50 mM NADPH or a regenerating system (made up in water just before starting the reactions)
- Stock solution of test drug/substrate**,** Example: 1 mM methoxyresorufin (Molecular Probes, Eugene, Oregon) in DMSO[a]
- Stock solution of 1 mM resorufin; a suitable dilution of this stock will need to be made for an internal standard (usually 0.5-10 µM in DMSO, depending on fluorometer sensitivity)
- Distilled deionized water
- Fluorometer with temperature-controlled cuvette housing

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 not, as yet, have 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 CYP1A1 and CYP1A2[1]. Unlike most other P450 assays, the O-dealkylation can be monitored continuously by fluorescence detection of the product, resorufin. The buffer type and concentration can dramatically affect the rate of O-dealkylation, and sodium/potassium phosphate is generally the buffer of choice for microsomal incubations.[b]

1. Prepare NADPH and test drug/substrate stock solutions.
2. Thaw enzyme and buffer mixes rapidly and place on ice.
3. Combine the following to prepare a buffer/substrate premix solution (enough for ten 100 µL reactions):

Water	768 µL
CYP1A2 Buffer Mix	126.7 µL
1 mM methoxyresorufin	5.3 µL
Total volume	950 µL

Pre-incubate the buffer/substrate solution at 37°C.

[a] *To prevent inhibition of the reaction, the final methanol concentration should not go beyond 1% .*

[b] *CYP1A2 may precipitate out of solutions with potassium phosphate concentrations of < 100 mM.*

4. For a 100 µl reaction, combine the following:

Buffer/substrate premix solution	90 µL
CYP1A2 Enzyme Mix	5 µL
Total volume	95 µL

   Using a temperature-controlled (37°C) fluorometer, monitor the change in fluorescence (excitation 530 nm, emission 585 nm) over the next minute. The change should be negligible.
5. Start the reaction by adding 5 µL of NADPH stock solution.
6. Record the change in fluorescence over time.
7. Once the reaction rate is observed to be linear, add 5 µL of resorufin as an internal standard. The fluorescence increase can be calibrated using the internal standard. The amount of resorufin added will depend on the sensitivity of the instrument and the amount of protein in the reaction.
8. Note that one can perform this assay by determining an endpoint rather than monitoring fluorescence continuously.[15]

4.0 REFERENCES

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