XENOTECH
A DIVISION OF SEKISUI

Product Instruction

Reaction Phenotyping Kit (H0500)

(Patent No. 5,478,723)

XenoTech's patented Reaction Phenotyping Kit (RPK) is designed to identify the human liver CYP and UGT enzyme(s) responsible for metabolizing a drug (or other xenobiotic), in order to predict pharmacokinetic variability, which can occur when a drug is metabolized by a polymorphically-expressed CYP or UGT enzyme. Reaction phenotyping (enzyme mapping) also provides valuable information on the potential for drug-drug interactions. Samples in the kit are carefully selected to minimize correlations or outliers that can interfere with reliable results.

Contents

- 16 individual samples of human liver microsomes
- 2 vials of pooled human liver microsomes
- Cytochrome P450 and UDP-glucuronosyltransferase

Caution

The Reaction Phenotyping Kit contains human-derived material. XenoTech accepts only non-transplantable tissue from donors who test negative for HIV 1 and 2, HTLV, and Hepatitis B and C. However, as a precaution, all human-derived samples should be regarded as a potential biohazard and should be stored, handled and discarded accordingly.
The Reaction Phenotyping Kit is intended for *in vitro* use only.

Storage

The Reaction Phenotyping Kit should be stored in an ultra-low freezer (-70°C or colder). At ultra-low temperatures, XenoTech's human liver microsomes are stable for >7 years.

Freezing and thawing samples

XenoTech's human liver microsomes can be frozen and thawed as many as ten times with no apparent loss of P450 activity, as reported by Pearce *et al.*

R Pearce, CJ McIntyre, A Madan, U Sanzgiri, AJ Draper, P Bullock, DC Cook, LA Burton, J Latham, C Nevins and A Parkinson. Effects of freezing, thawing, and storing human liver microsomes on cytochrome P450. *Arch. Biochem. Biophys*, 331, 145-169, 1996

Consequently, after an aliquot of human liver microsomes is taken from a vial, the residual sample can be re-frozen and used at a later date. Care should be taken to keep thawed samples on ice (-4°C), and to return them as quickly as possible to an ultra-low freezer for storage.

Experimental approaches to reaction phenotyping

Four *in vitro* approaches have been developed for reaction phenotyping - correlation analysis, chemical inhibition, antibody inhibition, and metabolism by recombinant human CYPs. Each has its advantages and disadvantages, and a **combination** of approaches is essential to identify the P450 enzyme(s) primarily responsible for metabolizing a drug, new molecular entity, or any other xenobiotic.

Suggested incubation conditions

For drug metabolism studies, liver microsomes can be incubated with a drug under a variety of conditions, therefore, the experimental conditions described below are provided simply as a guide:
Reactions are typically carried out in 200µL incubation mixtures that contain the following components at the final concentrations indicated in parentheses:

- Liver microsomes (0.1-0.2mg/mL)
- Substrate (drug or test article under investigation; various concentrations)
- Potassium phosphate buffer (50mM, pH 7.4)
- Magnesium chloride (3.0mM)
- EDTA (1.0mM, pH 7.4)
- Glucose-6-phosphate (5.0mM, pH 7.4)
- Glucose-6-phosphate dehydrogenase (1.0 Unit/mL)
- NADP (1.0mM, pH 7.4)

Liver microsomes are thawed and dispensed at ~4°C. The substrate, phosphate buffer, $MgCl_2$ and EDTA are typically combined and dispensed as a single solution, at ~4°C. The substrate may need to be dissolved in organic solvent. Because organic solvents can inhibit P450 enzymes, the amount of organic solvent should be kept to a minimum (less than 10 µL/mL or 1% of the incubation volume). If the substrate must be added in organic solvent, it should NOT be added to the microsomes directly because high concentrations of solvent can denature cytochrome P450. Substrates dissolved in organic solvents should be either diluted with buffer/$MgCl_2$/EDTA solution or added after these components to avoid exposing the microsomes to high concentrations of organic solvent. The last three components (NADPH-generating system), can also be combined and added as a single solution. Alternatively, the three components of the NADPH-generating system can be replaced with NADPH, although this is relatively expensive.

Incubations are typically conducted at 37°C, and are stopped with a denaturant, typically organic solvent or acid. If necessary, precipitated protein is pelleted in a bench-top centrifuge, and the clear supernatant fraction is analyzed (e.g., by LC/MS/MS) for metabolites and/or remaining substrate.

It is desirable to measure the metabolism of a substrate under initial rate conditions. These conditions must be determined experimentally by varying the amount of microsomal protein and incubation time to ascertain whether metabolite formation is directly proportional to time and protein concentration.

Occasionally, there is little or no information on the metabolism of the substrate with which to develop an analytical procedure. In such cases, it may be useful to incubate a fairly high concentration of pooled microsomal protein (e.g., 1mg/mL) with a high concentration of substrate (e.g., 100 µM or higher depending on solubility) for various times (e.g., 0, 5, 10, 15, 30, 45 and 60 minutes) in order to generate sufficient quantities of metabolites for detection purposes. It should be emphasized however, that reaction phenotyping should, if at all possible, be conducted with pharmacologically relevant concentrations of substrate under initial rate conditions. It will be necessary to reevaluate the effects of protein concentration and incubation time on rates of metabolite formation if the concentration of substrate is decreased in subsequent experiments. The sum of all metabolites should constitute less than 20% of the amount of substrate

present. Ideally, the amount of substrate consumed during the reaction should be less than 10% in order to measure initial rates of metabolite formation. However, it should be noted that in the case of substrate loss based studies, substrate loss of 20%-40% is targeted.

Once an analytical procedure has been developed and initial rate conditions have been established with the pooled sample of microsomes, the individual samples of microsomes can be examined for their ability to metabolize the compound of interest. Differences in the rates of formation of the drug metabolites are compared with the sample-to-sample variation in CYP, FMO3, and/or UGT activity (based on the information provided with the kit) either by simple regression analysis (r^2 = coefficient of determination) or by Pearson's product moment correlation analysis (r = correlation coefficient), where the marker CYP/FMO/UGT enzyme activity is the independent variable and the rate of formation of drug metabolite is the dependent variable. The latter determination also provides a measure of the statistical significance of any correlations. A high correlation usually identifies the P450 enzyme responsible for generating each metabolite.

Statistically significant correlations should always be confirmed with a visual inspection of the graph because there are two situations that can produce a misleadingly high correlation coefficient: (1) the regression line does not pass through or near the origin, and (2) there is an outlying data point that skews the correlation analysis.

Correlation analysis works particularly well when a single enzyme dominates the formation of a particular metabolite. When two or more CYP enzymes contribute significantly to the metabolism of a drug at pharmacologically relevant concentrations, the identity of the enzymes involved can be assessed by multivariate regression analysis. This approach successfully identifies the enzymes involved when each enzyme contributes 25% or more to metabolite formation, but it will likely not identify an enzyme that contributes only ~10%.

For additional help or information with this product please contact a customer service representative at **913.438.7450.**