

BD-Supersomes™, cDNA-Expressed Cytochrome P450 Enzymes

Catalog Numbers:

Human Cytochromes P450

456202, 456203, 456204, 456206, 456207, 456209, 456210, 456211,
456212, 456217, 456218, 456219, 456220, 456221, 456222, 456235,
456237, 456240, 456242, 456252, 456254, 456255, 456256, 456258,
456259, 456260, 456264, 456272, 456273, 456274, 456275

Animal Cytochromes P450

456501, 456502, 456503, 456505, 456510, 456511,
456513, 456514, 456517, 456519, 456521, 456531,
456532, 456533, 456536, 456612, 456621

Control Microsomes

456200, 456201, 456299, 456244, 456514

Guidelines for Use

FOR RESEARCH USE ONLY

NOT FOR USE IN DIAGNOSTIC PROCEDURES

BD-Supersomes™, cDNA-Expressed Cytochrome P450 Enzymes

Introduction

Cytochromes P450 comprise a membrane-bound superfamily of oxidative hemoprotein enzymes which are expressed in many tissues *in vivo* but are present at the highest level in mammalian liver. These enzymes have been found to be the principle route of metabolism for many hormones, drugs and other xenobiotics. The catalytic activity of cytochrome P450 enzymes requires interaction with its redox partner, NADPH:cytochrome P450 oxidoreductase (OR). Another hemoprotein, cytochrome b₅, can stimulate the catalytic activity of some cytochromes P450 for some substrates.

The metabolism of drugs and other compounds by enzymes can be studied using a variety of systems including isolated primary cells (e.g. hepatocytes), tissue fractions (e.g. microsomes and S9) and using cDNA-expressed enzymes. BD-Supersomes™ are a well-established source of cDNA-expressed enzymes for studies of metabolism.

This *Guidelines for Use* document provides information intended to aid in experimental design. It contains three parts: (1) a discussion of the major components of the assay and the potential influence on assay results, (2) a suggested general assay procedure and (3) further considerations for specific applications.

(1) Components of the Assay

Enzyme: Consult the product insert (batch data sheet) for important product information including cytochrome P450 content (expressed as pmole enzyme per mL of product), protein content (expressed as mg protein per mL of product), level of OR activity (expressed as nmole cytochrome c reduced per mg protein per minute) and activity for a known substrate of the expressed cytochrome P450. All BD-Supersomes should be stored at -80°C and thawed rapidly in a 37°C water bath and then stored on wet ice prior to use. Information regarding stability to freeze thaw cycles is also provided on the batch data sheet. BD recommends that if all the material is not to be used at once, that aliquots be prepared to minimize freeze thaw cycles and any potential variability associated with freeze thaw.

Enzyme concentration (pmole of enzyme per mL of incubation) is an experimental variable and will vary depending on the application and the susceptibility of the substrate to cytochrome P450 metabolism. In the absence of any other information, an enzyme concentration of 20 to 50 pmole/mL is a good starting point. Once some data are obtained, it may be desirable to adjust upward the concentration of enzymes which are more abundant in human liver (e.g. CYP3A's and CYP2C9) and adjust downward the concentration of enzymes which are less abundant in human liver (e.g. CYP2B6, CYP2C19 and CYP2D6). BD-Supersomes may be diluted in buffer prior to addition if needed.

Control BD-Supersomes¹ can be used to standardize the protein concentration and control this potential source of variability. Parallel incubations with control BD-Supersomes are also needed to control for any metabolism by enzymes native to the cell line used to express the cytochrome P450. If there is the potential for the compound being analyzed to be metabolized by OR, use of a control preparation with OR only is recommended (BD Biosciences Cat. Nos. 456244 and 456514).

¹ Most BD-Supersomes are expressed in the BTI-TN-5B1 cell line. However, a limited number of cytochrome P450s do not function well when expressed in this cell line and are expressed in SF9 cells. Use the Control which is prepared from the same cell line as the cytochrome P450 of interest.

Note: at very low protein concentrations (>20 ug per mL) a significant fraction of the enzyme may adhere to the walls of the vessel and lower than expected enzyme activity may be observed.

Buffer: Cytochromes P450 are active in a range of buffers of different concentrations. The product insert contains the buffer composition for the QC assay. This is a buffer which BD has found to function well with the specific cytochrome P450 product. Generally, 50 mM to 100 mM Tris HCl (pH 7.5) or potassium phosphate (pH 7.4) work well and these can be prepared by diluting BD Biosciences Cat. Nos. 451202 (0.5M Tris Buffer) or 451201 (0.5 M phosphate buffer). The activity of cytochromes P450 can vary with buffer and its ionic strength. When evaluating buffers it may be advisable to test new compositions relative to the buffer used for the QC assay.

NADPH: NADPH cofactor (or some other source of reducing equivalents) must be supplied for cytochrome P450 activity. We recommend the use of an NADPH generating system which can be prepared from a 1:20 dilution of BD Biosciences Cat. No. 451220 (Solution A) and a 1:100 dilution of BD Biosciences Cat. No. 451200 (Solution B). An NADPH solution can also be added directly. Typically a concentration around 1 mM is used to mitigate depletion of this cofactor during longer incubations.

Vessel: A variety of vessels and materials can be used with polypropylene and glass being most commonly used (we recommend polypropylene). Polystyrene plates are commonly used for some assay applications. You should check for compatibility with any organic solvents (e.g. stop solutions).

Solvent: Cytochromes P450 are well known to be inhibited by a variety of organic solvents and the impact of this inhibition will vary depending on the application. If DMSO is to be used, a solvent concentration of 0.2% or less is recommended. Acetonitrile is tolerated by most human cytochrome P450s at concentrations up to 2%.

Substrate Concentration: The tested substrate concentration will vary depending on the application. If metabolic stability is being measured, it is customary to use a low substrate concentration (e.g. 1 uM) based on an assumption that this is well below the apparent K_m and the observed rate approximates the Intrinsic Clearance². If inhibition of the cytochrome P450 is being measured, it is customary to use a substrate concentration which is near the apparent K_m value as this allows a more facile estimation of the apparent K_i from an IC_{50} . If metabolite formation is being measured for reaction phenotyping or kinetic parameters are being determined, a range of substrate concentrations (above and below the apparent K_m value) may be needed.

Assay Linearity: Information regarding assay linearity with respect to enzyme concentration and incubation time is provided on the batch data sheet. The degree of linearity will vary among substrates and should be determined experimentally for new substrates.

Order of Addition of Assay Components: The combination of substrate, enzyme and NADPH will cause metabolism to begin. We recommend initiating metabolism by pre-warming the substrate, buffer and NADPH to 37°C and then adding cold BD-Supersomes in a small volume of buffer. An alternative approach is to pre-warm the enzyme, substrate and buffer and initiate metabolism by the addition of NADPH. You may wish to compare these two approaches to determine which works best for your specific assay.

Agitation: After an initial mixing (e.g. by pipetting, inverting a sealed tube or gentle vortexing) no further agitation is typically needed. Vigorous agitation may inactivate the cytochrome P450.

Stop Solutions: An example stop solution is provided in the batch data sheet QC assay. The stop solution serves two purposes: to inactivate the cytochrome P450 and to precipitate the protein so it does not interfere with metabolite analysis. A 0.5x to 2x volume of acetonitrile is commonly used as a stop solution. Acidification of the stop solution with acetic acid (or some

² Intrinsic Clearance is the ability of the liver to remove a drug absent other, confounding factors. In *in vitro* assays it is defined as the V_{max} divided by the K_m .

other acid) may be needed to control the ionization state of the substrate and metabolite (e.g. for chromatography or mass spectrometry). Protein is typically removed by centrifugation (e.g. 10,000 x g for 3 minutes in microcentrifuge tubes or 4000 x g for 20 minutes in multiwell plates).

Metabolite Analyses: A basic method for metabolite analysis by HPLC separation, fluorometric or spectrophotometric detection is provided in the batch data sheet. The analytical method should be adapted based on the metabolite(s) to be detected.

(2) Suggested General Assay Procedure

- I. Thaw BD-Supersomes (1000 pmole per mL), NADPH Regenerating System Solutions A & B and keep on wet ice.
- II. Prepare 1 mM Substrate in acetonitrile. Store appropriately based on substrate stability.
- III. Also needed, 0.5 M potassium phosphate pH 7.4 (BD Biosciences Cat. No. 451201), acetonitrile, 1.7 mL microcentrifuge tubes, pipettors and 37°C water bath.
- IV. Combine the following
 - i. 710 uL purified water
 - ii. 200 uL 0.5 M Potassium phosphate pH 7.4 (BD Biosciences Cat. No. 451201, 100 mM final concentration)
 - iii. 50 uL NADPH Regenerating System Solution A (BD Biosciences Cat. No. 451220)
 - iv. 10 uL NADPH Regenerating System Solution B (BD Biosciences Cat. No. 451200)
 - v. 10 uL Substrate in acetonitrile (10 uM final concentration)
- V. Warm to 37°C for 5 minutes in a water bath.
- VI. Initiate by the addition of 20 uL (20 pmole) BD-Supersomes. Mix by inverting the capped tube twice. Return to the 37°C water bath.
- VII. After 0, 5, 10, 20, 30, 40, 50 and 60 minutes, withdraw 100 uL from the incubation and add to 100 uL acetonitrile. Mix and place on wet ice.
- VIII. Centrifuge 10,000 x g (or higher) for 3 minutes.
- IX. Withdraw the supernatant from the protein pellet.
- X. Analyze according to your analytical method.

(3) Further Considerations for Specific Applications

- For metabolic stability determinations, keep the substrate concentration low (e.g. 1 uM).
- When determining the linearity with respect to time and protein concentration, it is recommended to use a range of enzyme concentrations (spanning 5 to 100 pmole per mL) and a range of incubation times (1 minute to 60 minutes). A range finding study may be helpful to design a definitive experiment.
- When determining enzyme kinetic parameters (e.g. apparent K_m and V_{max}), use 10 to 20 substrate concentrations spanning the expected apparent K_m . In most cases, the apparent K_m will be between 1 uM and 100 uM.
- When conducting inhibition experiments (e.g. determination of an IC_{50}) use a substrate concentration near the apparent K_m .
- Avoid excessive substrate depletion (e.g. >20% substrate consumption) in the determination of kinetic parameters and inhibition experiments.
- Consider the amount of specific cytochromes P450 in pooled HLM (e.g. see characterization data for UltraPool™ HLM 150) when designing experiments to compare metabolism by BD-Supersomes relative to HLM.