BD-Supersomes™, cDNA-Expressed UDP-Glucuronosyl Transferase Enzymes

Catalog Numbers:

Human UDP-Glucuronosyl Transferase Enzymes
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Human UGT Insect Control
456400

Guidelines for Use

FOR RESEARCH USE ONLY

NOT FOR USE IN DIAGNOSTIC PROCEDURES

BD-Supersomes™, cDNA-Expressed UDP-Glucuronosyl Transferase Enzymes

Introduction

UDP-Glucuronosyl transferase enzymes (UGTs) comprise a membrane-bound superfamily of conjugative enzymes which are expressed in many tissues *in vivo* but are present at the highest level in mammalian liver and intestine. These enzymes conjugate the substrate with glucuronic acid which is supplied to the enzyme in the higher energy form UDP-glucuronic acid (UDPGA). This conjugation typically occurs at a free hydroxyl, carboxyl or amine functional group on the substrate. UGT enzymes have been found to be a principle route of metabolism for many hormones, drugs and other xenobiotics. In many cases, UGT metabolism follows cytochrome P450 metabolism which creates the functional group for UGT conjugation.

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. While UGTs in mammalian liver tissue fractions are known to exhibit significant "latency" in their catalytic activity[1], we have observed that UGTs in BD-Supersomes exhibit lower level of latency than in mammalian tissue fractions.

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 protein content (expressed as mg protein per mL of product) and activity for a known substrate of the expressed UGT. 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 (mg enzyme per mL of incubation) is an experimental variable and will vary depending on the application and the susceptibility of the substrate to UGT metabolism. In the absence of any other information, an enzyme concentration of 0.4 to 0.8 mg/mL is a good starting point. Once some data are obtained, it may be desirable to adjust upward or downward the concentration of enzyme.
Control BD-Supersomes[2] 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 UGT.
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: UGTs have generally been found to be more active in Tris buffers relative to phosphate buffers. A recommended UGT Reaction Mix, containing optimal concentrations of Tris buffer (pH 7.5), alamethicin and $MgCl_2$ is available from BD Biosciences (Cat. No. 451320, 5x concentrate). When evaluating new buffers it may be advisable to test new compositions relative to the buffer used for the QC assay.

Alamethicin Activation (latency): The pore forming agent, alamethicin, or detergents can be used to overcome the latency phenomena associated with UGT enzymes. Alamethicin is the preferred activating agent because it is non-denaturing to UGT or CYP isoforms at excess

[1] Fisher, M.B. et al., Drug Metab. and Disp. 28 (2000) 560-566.
http://dmd.aspetjournals.org/content/28/5/560.abstract
[2] Human UGT Insect Control, BD Bioscience Cat. No. 456400.

concentrations. Maximal stimulation by alamethicin can be obtained at 25 ug/mL over a broad range of UGT or HLM protein concentrations. Pre-incubating alamethicin with UGT or HLM protein prior to initiating the reaction is not necessary to achieve maximal stimulation.

UDPGA: UPDGA cofactor must be supplied for UGT activity. We recommend use of an UDPGA cofactor system which can be prepared from a 1:12.5 dilution of BD Biosciences Cat. No. 451300 (Solution A, 25 mM UDPGA) to result in a final UDPGA concentration of 2 mM, a saturating concentration. UDPGA concentration can be an experimental variable. Different UGT enzymes have differing affinities for UDPGA.

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: UGTs are susceptible to inhibition by a variety of organic solvents and the impact of this inhibition will vary depending on the application. As a general rule, DMSO and methanol are least inhibitory and can be used at final concentration of up to 2% little impact on activity. If acetonitrile is to be used, a solvent concentration of 1% or less is recommended.

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 Clearance[3]. If inhibition of the UGT 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 UDPGA will cause metabolism to begin. We recommend initiating metabolism by pre-warming the substrate, UGT reaction mix (Tris buffer, $MgCl_2$, alamethicin) and UDPGA to 37^oC and then adding cold BD-Supersomes in a small volume of buffer. An alternative approach is to pre-warm the enzyme, substrate and UGT reaction mix and initiate metabolism by the addition of UDPGA. 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 UGT.

Stop Solutions: An example stop solution is provided in the batch data sheet QC assay. The stop solution serves two purposes: to inactivate the UGT 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 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. For LC/MS analysis, be aware of in-source fragmentation of the glucuronides yielding peaks that mimic the response of the parent molecule.

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[3] 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.

(2) Suggested General Assay Procedure

I. Thaw BD-Supersomes (5 mg protein per mL), UGT Reaction Mix Solutions A & B and keep on wet ice.
II. Prepare 5 mM Substrate in DMSO. Store appropriately based on substrate stability.
III. Also needed acetonitrile, 1.7 mL microcentrifuge tubes, pipettors and 37°C water bath.
IV. Combine the following
    i. 678 uL purified water
    ii. 200 uL UGT Reaction Mix Solution B (BD Biosciences Cat. No. 451320)
    iii. 80 uL UGT Reaction Mix Solution A (BD Biosciences Cat. No. 451300)
    iv. 2 uL Substrate in DMSO (10 uM final concentration)
V. Warm to 37°C for 5 minutes in a water bath.
VI. Initiate by the addition of 40 uL (0.2 mg) 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.

Final reagent concentrations in reaction mix:

Reagent	Final Concentration
Tris-HCl (pH 7.5)	50 mM
$MgCl_2$	8 mM
Alamethicin	25 ug/ml
UDPGA	2 mM

(3) Further Considerations for Specific Applications

- When establishing a new assay, always include parallel incubations with UGT control BD-Supersomes
- 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 0.1 to 0.8 mg 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 above 10 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.