

Mammalian Liver Cytosol

Catalog Numbers:

Human Liver Derived
452115, 452861

Animal Liver Derived
452581, 452461, 452462

Guidelines for Use

FOR RESEARCH USE ONLY

NOT FOR USE IN DIAGNOSTIC PROCEDURES

Mammalian Liver Cytosol

Introduction

Mammalian liver is the principal organ for drug and other foreign compound (xenobiotic) metabolism. Cytosol is prepared by centrifugation from a crude tissue (liver) homogenate at 100,000 x g. The supernatant is referred to as "cytosol¹." and the protein concentration adjusted to 20 mg/mL. Cytosol is comprised of soluble proteins and contains N-acetyl transferase (NAT) enzymes, sulfotransferase (SULT) enzymes, other proteins and enzymes.

Each lot of material is characterized for the level of activity for a series of NAT, SULT and/or other enzymes. Use of these products to study metabolism requires fortification with the appropriate cofactors for the enzyme class to be studied. NAT activity requires fortification with Acetyl-Coenzyme A (Acetyl-CoA) and SULT activity requires fortification with adenosine 3'- phosphate 5'-phosphosulfate (PAPS).

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 procedures 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 (typically 20 mg/mL using the method of Lowry), level of various enzyme activities. All mammalian liver cytosol should be stored at -80°C and thawed rapidly in a 37°C water bath and then stored on wet ice prior to use. 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.

Protein concentration is an experimental variable and will vary depending on the application and the susceptibility of the substrate to metabolism. In the absence of any other information, an enzyme concentration of 1 mg/mL is a good starting point.

Buffer: Cytosolic enzymes 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 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). When evaluating new buffers it may be advisable to test new compositions relative to the buffer used for the QC assay.

Acetyl-CoA: NAT function requires a source of acetyl-CoA. BD recommends using an acetyl-CoA generating system. This system is comprised of: 0.1 mM (81 ug per mL) acetyl-CoA, 4.6 mM (1.1 mg/mL) acetyl-d,l-carnitine hydrochloride and 0.06 units/mL carnitine acetyl transferase).

PAPS: SULT function requires a source of 3'-Phosphoadenosine-5'-phosphosulfate (PAPS). BD recommends adding 0.1 mM PAPS to the incubation.

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: Drug metabolizing enzymes are well known to be inhibited by a variety of organic solvents and the impact of this inhibition will vary depending on the application. Consult the scientific literature for what is known for the enzyme classes being studied.

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

¹ The material in BD Biosciences cytosol products are subjected to a second 100,000 x g centrifugation and the supernatant from this second centrifugation is isolated, assayed and packaged. The purpose of this second centrifugation is to reduce any potential contamination with microsomal materials.

concentration (e.g. 1 μM) 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 enzyme is being measured, it is customary to use a substrate concentration which is near the apparent K_m value as this allows easier 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: 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 cofactors (acetyl-CoA and/or PAPS) will cause metabolism to begin. We recommend that to initiate metabolism by pre-warming the substrate, buffer and cofactors to 37°C and then adding cold liver cytosol 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 cofactors. 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 vortexing) no further agitation is typically needed.

Stop Solutions: An example stop solution is provided in the batch data sheet QC assay. The stop solution serves two purposes: to inactivate the enzymes 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.

(2) Suggested General Assay Procedures

SULT Enzymes

- I. Thaw liver cytosol (20 mg protein per mL)
- 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
 - 723 μL purified water
 - 200 μL 0.5 M Tris (pH 7.5) BD Biosciences Cat. No. 451202
 - 50 μL 2 mM (1.01 mg per mL) PAPS (lithium salt)
 - 2 μL Substrate in DMSO (10 μM final concentration)
- V. Warm to 37°C for 5 minutes in a water bath.
- VI. Initiate by the addition of 25 μL (0.5 mg) of cytosol. 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 μL from the incubation and add to 200 μL 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.

NAT Enzymes

- I. Thaw liver Cytosol (20 mg protein per mL).
- 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
 - 170 μL purified water

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 .

- 500 μ L 100 mM (14.9 mg per mL) triethanolamine (pH 7.5) in purified water
 - 100 μ L 1 mM (0.81 mg per mL) acetyl-CoA in purified water
 - 200 μ L 23 mM (5.51 mg per mL) acetyl-d,l-carnitine in purified water
 - 2 μ L 500 mM (186 mg per mL) disodium EDTA dihydrate
 - 1 μ L 1 M (154 mg per mL) Dithiothreitol (DTT)
 - 0.6 units carnitine acetyl transferase (e.g. Sigma Cat. No. C4899)
 - 2 μ L Substrate in DMSO (10 μ M final concentration)
- V. Warm to 37°C for 5 minutes in a water bath.
- VI. Initiate by the addition of 25 μ L (0.5 mg) of cytosol. 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 μ L from the incubation and add to 200 μ L 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 μ M).
- When determining the linearity with respect to time and protein concentration, it is recommended to use a range of protein concentrations (0.2 to 2 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 for NATs and SULTs will be between 1 μ M and 100 μ M. 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.