

Mammalian Liver S9

Catalog Numbers:

Human Liver Derived  
452116, 452961

Animal Liver Derived  
452491, 452494, 452591, 452593, 452594, 452693, 452791, 452792,

*Guidelines for Use*

FOR RESEARCH USE ONLY

NOT FOR USE IN DIAGNOSTIC PROCEDURES

## Mammalian Liver S9

Introduction

Mammalian liver is the principal organ for drug and other foreign compound (xenobiotic) metabolism. S9 fractions are prepared by centrifugation from a crude tissue (liver) homogenate. Tissue is homogenized in buffer and subjected to a 9000 x g centrifugation. The supernatant from this centrifugation is recovered, designated "S9" and the protein concentration adjusted to 20 mg/mL. S9 is comprised of microsomes<sup>1</sup> which are principally derived from the membranes of the endoplasmic reticulum and soluble proteins (or cytosol). Microsomal membranes provide an enriched source of membrane bound drug metabolizing enzymes. Principal among these are the cytochrome P450 (CYP) superfamily of oxidative hemoprotein enzymes and uridine glucuronosyl transferase (UGT) enzymes but many other enzymes are present. The cytosolic component 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 CYP, UGT and/or other enzymes. Use of these products to study metabolism requires fortification with the appropriate cofactors for the enzyme class to be studied. In the case of CYPs, fortification with NADPH is recommended. In the case of UGTs, fortification with uridine diphosphate glucuronic acid (UDPGA) is recommended. UGTs demonstrate the property of "latency" in microsomes. Latency is believed to be due to restricted access of the substrate and/or cofactors to the UGT active site<sup>2</sup>. Pore forming agents such as alamethicin are used to mitigate the latency of UGT enzymes<sup>3</sup>. 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 S9 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:** CYPs and UGTs 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). The activity of CYPs and UGTs can vary with buffer and its ionic strength. UGTs have generally been found to be more active in Tris buffers relative to phosphate buffers. A recommended UGT buffer, containing alamethicin 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.

**NADPH:** NADPH cofactor (or some other source of reducing equivalents) must be supplied for CYP 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.

**UDPGA:** UDPGA 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. UDPGA concentration can be an experimental variable. Different UGT enzymes have differing affinities for UDPGA.

**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 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 concentration (e.g. 1  $\mu$ M) based on an assumption that

<sup>1</sup> Membranes from other cellular organelles are typically present in these relatively crude tissue fractions.

<sup>2</sup> The active site of UGTs is generally present in the luminal side of the endoplasmic reticulum which becomes the interior of the microsomes upon homogenization. In contrast, CYPs are present on the opposite side of the ER and have freer access to substrate and cofactors.

<sup>3</sup> Fisher, M.B. et al., *Drug Metab. and Disp.* 28 (2000) 560-566. <http://dmd.aspetjournals.org/content/28/5/560.abstract>

this is well below the apparent  $K_m$  and the observed rate approximates the Intrinsic Clearance<sup>4</sup>. 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 (NADPH, UDPGA, acetyl-CoA and/or PAPS) will cause metabolism to begin. We recommend initiating metabolism by pre-warming the substrate, buffer and cofactors to 37°C and then adding cold liver microsomes 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

### Cytochrome P450 and other NADPH Dependent Enzymes present in S9

- I. Thaw liver S9 (20 mg protein per mL), NADPH Regenerating System Solutions A & B and keep on wet ice.
- II. Prepare 5 mM Substrate in dimethylsulfoxide (DMSO). 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
  - 688 uL purified water
  - 200 uL 0.5 M Potassium phosphate pH 7.4 (BD Biosciences Cat. No. 451201, 100 mM final concentration)
  - 50 uL NADPH Regenerating System Solution A (BD Biosciences Cat. No. 451220)
  - 10 uL NADPH Regenerating System Solution B (BD Biosciences Cat. No. 451200)
  - 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 50 uL (1 mg) liver S9. 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.

### UGT Enzymes

- I. Thaw liver S9 (20 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
  - 688 uL purified water
  - 200 uL UGT Reaction Mix Solution B (BD Biosciences Cat. No. 451320)
  - 80 uL UGT Reaction Mix Solution A (BD Biosciences Cat. No. 451300)
  - 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 50 uL (1 mg) liver S9. 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, withdrawn 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.

### SULT Enzymes

- I. Thaw liver S9 (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.

<sup>4</sup> 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  $K_m$ .

- IV. Combine the following
  - 723  $\mu$ L purified water
  - 200  $\mu$ L 0.5 M Tris (pH 7.5) BD Biosciences Cat. No. 451202
  - 50  $\mu$ L 2 mM (1.01 mg per mL) PAPS (lithium salt)
  - 2  $\mu$ L Substrate in DMSO (10  $\mu$ M final concentration)
- V. Warm to 37°C for 5 minutes in a water bath.
- VI. Initiate by the addition of 25  $\mu$ L (0.5 mg) of S9. 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  $\mu$ L from the incubation and add to 200  $\mu$ 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.

**NAT Enzymes**

- I. Thaw liver S9 (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  $\mu$ L purified water
    - 500  $\mu$ L 100 mM (14.9 mg per mL) triethanolamine (pH 7.5) in purified water
    - 100  $\mu$ L 1 mM (0.81 mg per mL) acetyl-CoA in purified water
    - 200  $\mu$ L 23 mM (5.51 mg per mL) acetyl-d,l-carnitine in purified water
    - 2  $\mu$ L 500 mM (186 mg per mL) disodium EDTA dihydrate
    - 1  $\mu$ L 1 M (154 mg per mL) Dithiothreitol (DTT)
    - 0.6 units carnitine acetyl transferase (e.g. Sigma Cat. No. C4899)
    - 2  $\mu$ L Substrate in DMSO (10  $\mu$ M final concentration)
  - V. Warm to 37°C for 5 minutes in a water bath.
  - VI. Initiate by the addition of 25  $\mu$ L (0.5 mg) of S9. 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  $\mu$ L from the incubation and add to 200  $\mu$ 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  $\mu$ 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 (e.g. 0.1X to 10X) the expected apparent  $K_m$ . In most cases, the apparent  $K_m$  for NATs and SULTs will be between 1  $\mu$ M and 100  $\mu$ 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.