HEPATOPAC® Kit

Metabolic Stability Protocol

Use of HEPATOPAC Cultures for Determination of Hepatic Drug Clearance

TP-005 V3.0

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Product Description

This instruction manual describes use of HEPATOPAC Kits to conduct Metabolic Stability studies.

> NOTE:
> Read these instructions in their entirety before starting to unpack or perform any of the lab work described in this protocol.

Materials and Storage

Upon receipt of boxes, refer to the HEPATOPAC Maintenance Instructions inside Box A for instructions on unpacking HEPATOPAC kits and caring for HEPATOPAC cultures.

Kit Contents

Box(es) A: Store at -20°C

- HEPATOPAC Culture Media Components
- HEPATOPAC Maintenance Instructions and Application Protocol

Box(es) B: Store at 2-8°C

- HEPATOPAC Culture Media Components
- Additional Sterile Lids

Box(es) C: Store at 37°C/**10% CO_2** Incubator, humidified with full water pan ≥ 95%

- HEPATOPAC Plate(s)
- Stromal Only Plate(s) as applicable

Additional Required Equipment and Materials:

- Laminar Flow Biological Safety Cabinet (BSC), Class II
- Cell Culture Incubator, 37°C, **10% CO_2**, ≥ 95% humidity (FOR CULTURE MAINTENANCE)
- Cell Culture Incubator, 37°C, **5% CO_2**, ≥ 95% humidity (FOR APPLICATION)[1]
- 37°C Water Bath
- Phase Contrast Microscope with Digital Image Capture Accessories
- -80°C Freezer
- Refrigerator, 2-8°C Storage
- Pipette Aid
- Sterile Serological Pipettes (10 - 25 mL)
- Multichannel Pipette (Electronic or Manual)
- Micropipettes
- Sterile Micropipette Tips
- 50 mL Sterile Conical Tubes and appropriately sized Rack
- Sterile Reagent Reservoirs
- Sample Collection Supplies, i.e. 1-2 mL Deep Well Blocks, Cap Mats, Sealing Press
- Test Compound(s)
- Sterile DMSO (or other solvent for making compound stocks)
- Timer/Clock
- Dry Ice

[1] Metabolic Stability Application medium is buffered for 5% CO_2

Handling/Caution Statement

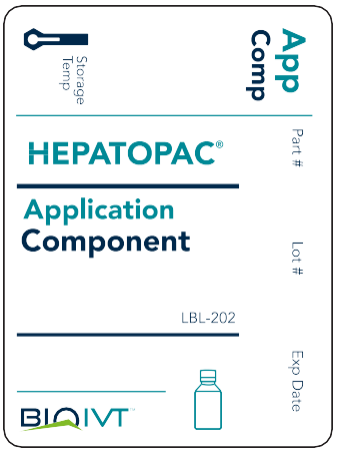
1. Do not use a vacuum-powered aspiration device with HEPATOPAC media.
2. Avoid scraping the monolayer with pipette tips. This may cause damage to the monolayer in that region of the well.

NOTE:
Prior to initiating a Metabolic Stability study using HEPATOPAC cultures, complete the Study Checklist (Appendix A) and refer to the Dosing Plate Map (Appendix B).

Protocol

Metabolic Stability: Application Medium Preparation

1. Locate the bottles labeled as follows:
   - Application Base Metabolic Stability
   - Application Component
2. Locate the vials labeled with a blue line that say, "App":



3. Prior to preparing the medium, thaw Component Part # 5030C in a 37°C water bath. Component Part # 5023C is stored at 2-8°C and does not need to be thawed. Component Part #s 5011C and 5012C are stored at -20°C and must be thawed at **room temperature** (not in a 37°C water bath).

4. Add the indicated volume of each Component (shown in Table 1 below) to the Application Base Metabolic Stability Bottle(s). Note the following:
   - Refer to the appropriate column for the volume of each component required for either the 125 mL bottle, 250 mL bottle, or the 500 mL bottle.
   - As each component is added, check off the appropriate box on the back of the Application Base Bottle(s).
   - After the Application Base bottle(s) has been reconstituted with the components, it is referred to (in this protocol) as "**Metabolic Stability Application Medium**". Label the bottle(s) appropriately. Store reconstituted medium at 2-8°C. Shelf life is 7 days.

NOTE:
The final volume in the 125 mL bottle will be 100 mL of Metabolic Stability Application Medium.
The final volume in the 250 mL bottle will be 250 mL of Metabolic Stability Application Medium.
The final volume in the 500 mL bottle will be 500 mL of Metabolic Stability Application Medium.

Table 1: Application Medium Component Volumes

Component Part Number	Volume to Add to Application Base in 125 mL Bottle	Volume to Add to Application Base in 250 mL Bottle	Volume to Add to Application Base in 500 mL Bottle
5030C	1.92 mL	4.8 mL	9.6 mL
5023C	3.9 mL	9.75 mL	19.5 mL
5011C	1.0 µL	2.5 µL	5.0 µL
5012C	1.0 µL	2.5 µL	5.0 µL

5. Return the Application Components and Metabolic Stability Application Medium to the appropriate storage conditions. (Refer to Step 3 above)

NOTE:
Initiate the Metabolic Stability application after allowing the HEPATOPAC plates to recover for two days at 37°C/**10% CO_2** after receipt of shipment. For example, if the shipment was received on Tuesday, allow the cultures to recover until Thursday. Cultures may be dosed starting on Thursday. Please review the HEPATOPAC Maintenance Instructions for information on care of plates upon receipt.
After recovery, plates will incubate at **5% CO_2** during performance of the application.

Metabolic Stability: Study Initiation

1. Warm the Metabolic Stability Application Medium (containing appropriate components as described in the section above entitled "Metabolic Stability: Application Medium Preparation) in a 37°C water bath for ≥ 30 minutes.
2. Wipe the Biosafety Cabinet (BSC) clean with ethanol.
3. Obtain the following supplies and place them in the BSC:
   - Reagent Reservoirs
   - Pipette Aid
   - Serological Pipettes (10-25 mL)

- 50 mL Conical Tubes and Rack
- Sterile Pipette Tips
- Pipette
- Pre-warmed Metabolic Stability Application Medium

NOTE:
BSL-2 practices must be followed during all work with HEPATOPAC cultures. Universal Precautions must be observed and all material should be treated as potentially infectious.

4. Carefully remove HEPATOPAC plates from the incubator. Observe the individual wells using a Phase Contrast Microscope. Cultures should have defined islands of hepatocytes surrounded by stromal cells, distinct nuclei and nucleoli, and a network of bile canaliculi as described in the HEPATOPAC Maintenance Instructions.
5. Carefully place the HEPATOPAC plates in the BSC.

NOTE:
Prior to initiating the study, a representative image of the cultures should be taken using a digital camera attached to the Phase Contrast Microscope.

Important Handling Requirements:
1. Do not use a vacuum-powered aspiration device with HEPATOPAC plates.
2. Avoid scraping the monolayer with pipette tips. This may cause damage to the monolayer in that region of the well.

6. Wash the wells once (1X) with Metabolic Stability Application Medium, including cell-free wells in rows A and H (see Appendix B).
   - To wash: Remove the maintenance medium and apply the appropriate species-specific volume of Metabolic Stability Application Medium to each well. Refer to Table 2 below for species-specific volumes.
   - Remove the Metabolic Stability Application Medium and re-fill the wells with the appropriate species-specific volume of fresh Metabolic Stability Application Medium.

Table 2: Species-specific Volumes per Well

Human	Rat	Monkey	Dog
64µL	50µL	64µL	64µL

NOTE:
If working with a multi-species plate, volumes may vary per well. Make sure the correct volume is added to the appropriate wells in the plate.

7. Pre-incubate the plates in a 37°C, **5% CO_2** incubator for 2-4 hours to allow cells to adapt to serum-free conditions.
8. During the pre-incubation, prepare compound dosing solutions.

Compound Dosing Solution Preparation

1. Obtain the following supplies and place them in the BSC:
   - Sterile DMSO
   - Compounds
   - 1-2 mL 96-well Deep Well blocks
2. Prepare a stock concentration in DMSO (or other appropriate solvent) of each test compound and control(s) at 200-1000X of the final concentration (i.e. 0.2 mM – 1 mM). Refer to Table 3 for reference compound information.
   - The standard Metabolic Stability dosing concentration is 1 µM.

NOTE:
For 1000X compound stocks, the final concentration of DMSO or solvent in the medium is 0.1%. If necessary, the percent of solvent may be increased, but must not exceed 0.5% or a 200x compound stock.

Reference Equations for Compound Stock Preparations

$$Mass\ (g) = Molarity\ \left(\frac{mol}{L}\right) * Volume\ (L) * Molecular\ Weight\ \left(\frac{g}{mol}\right)$$

$$Volume\ (L) = Mass(g) \div (Molecular\ Weight\ (\frac{g}{mol}) * Molarity\ (\frac{mol}{L}))$$

Table 3: Metabolic Stability Reference Compound Information

Parent Compound	Clearance Rate[2]	Tested Species	Suggested Collection Time Points
Diazepam	Low	Human	0, 4, 24, 48, 96 and 168 Hours
Tolbutamide	Low	Human	
Lorazepam	Low	Rat	

3. Prepare a 2X dosing solution of each compound in Metabolic Stability Application Medium and mix well. For example: To make a 2X stock from a 1000X stock, add 2 µL of 1000X compound stock to 998 µL of Application Medium.

Table 4: Volume of 2X Metabolic Stability Dosing Solution Required per Well

Human	Rat	Monkey	Dog
32µL	25µL	32µL	32µL

[2] For more information, refer to **Meeting the Challenge of Predicting Hepatic Clearance of Compounds Slowly Metabolized by Cytochrome P450 Using a Novel Hepatocyte Model, HepatoPac.** (2013). Drug Metab Dispos 41:2024-2032. doi:10.1124/dmd.113.053397

NOTE:
The final applied compound concentration will be 1X. 2X concentrations of compounds and vehicle control(s) are diluted with an equal volume of application medium when applied to the cultures during the dosing steps (Section entitled, "Compound Dosing").

A 2X dosing strategy allows the cultures to be continuously bathed in medium and prevents desiccation.

If working with a multi-species plate, volumes may vary per well. Make sure the proper volume is added to the appropriate wells in the plate.

4. Prepare a 2X solution of vehicle control by diluting the appropriate solvent in Metabolic Stability Application Medium at the same percentage used to prepare the 2X dosing solution of test compounds.

Compound Dosing

NOTE:
Only work with two plates at a time to prevent cultures from being held in minimal media for an extended period of time while dosing. Application of dosing solutions should follow the guidelines in the HEPATOPAC Maintenance Instructions.

1. Remove the medium from the wells and apply fresh Metabolic Stability Application Medium to each well at half the final dosing volume (see Table 5 below).
2. Apply an equal volume (see Table 5) of 2X dosing solution(s) to each well. Apply the 2X dosing solution to the latest time point wells first.

Table 5: Dosing Volumes per Well

Medium Type	Human	Rat	Monkey	Dog
Metabolic Stability Application Medium	32 µL	25 µL	32 µL	32 µL
2X Dosing Solution	32 µL	25 µL	32 µL	32 µL
Final Dosing Volume (1X)	64 µL	50 µL	64 µL	64 µL

NOTE:
Ensure that only the 0-hour time point is dosed in columns 1 and 12 to prevent exposure of later time points to edge well evaporation. (See Appendix B)

Dosing should be performed starting with the latest time point. Time 0 time points should be dosed last.

If working with a multi-species plate, volumes may vary per plate. Make sure the proper volume is added to the appropriate wells in the plate.

3. Gently swirl the plates and return them to the 37°C, **5% CO_2** incubator.
4. Record start time.
5. Monitor culture morphology throughout the application. Document morphology of cultures with phase contrast images.

Metabolic Stability: Sample Collection

NOTE:
The following protocol details the collection of culture supernatants and cell lysate collection/extraction. Depending on the study design and bioanalytical requirements, the use of a crashing solution and the collection of cellular material may be necessary to include in the sample collection protocol. Please contact BioIVT with any technical questions: customerservice@bioivt.com

1. Warm the Metabolic Stability Application Medium in a 37°C water bath for ≥ 30 minutes.
2. Obtain the following supplies and place in the BSC:
    - Sterile Pipette Tips
    - Pipette
    - Pre-warmed Metabolic Stability Application Medium
    - Sample collection supplies, i.e. 1-2 mL Deep Well Blocks, Cap Mats, Sealing Press
    - Timer
3. Properly label the collection block. Record the time of sample collection for each time point. Reference Appendix C for the plate template.
4. At each time point, follow the appropriate sample collection instructions below:
    a) Supernatant Collection
        (i) Supernatant, without crashing: Collect a consistent volume of the supernatant in shallow well (300 µL), polypropylene plates, unless otherwise specified.
            - Transfer 50 µL out of the initial 64 µL sample volume for human, monkey, and dog cultures.
            - Transfer 40 µL out of the initial 50 µL sample volume for rat cultures.
        (ii) Supernatant, with crashing: Collect a consistent volume of the supernatant in deep well (2 mL), polypropylene plates or tubes.
            - Transfer 50 µL out of the initial 64 µL sample volume for human, monkey, and dog cultures.
            - Transfer 40 µL out of the initial 50 µL sample volume for rat cultures.
            - Crash supernatant samples in the collection block(s) or tubes with ice cold acetonitrile (1X sample volume) plus applicable internal standard.
        (iii) If cell lysates will be collected, proceed to the step below: "Cell Lysate Extraction / Collection"
    b) Cell Lysate Extraction / Collection
        (i) From the culture wells collected above, remove residual supernatant.
        (ii) Wash culture wells 1X with PBS.

(iii) Apply extraction buffer directly to adhered cells in tissue culture plate (ice cold acetonitrile at 1X sample volume) plus applicable internal standard.
(iv) Scrape the cells using the tip of the collection pipette, scraping from side-to-side so as to release all cell components into the extraction solution.
(v) Transfer cell lysates / extracts to a separate deep well (2 mL) polypropylene plate or tube.

5. Replace the collected supernatant with the appropriate species-specific volume of fresh Metabolic Stability Application Medium (see Table 2) to maintain consistent humidity across the plate. **Wells should not be left dry**.
6. Use a Plate Sealing press to seal the collection block with a Cap Mat.
7. Store samples at -80°C until bioanalysis.

References

"Meeting the Challenge of Predicting Hepatic Clearance of Compounds Slowly Metabolized by Cytochrome P450 Using a Novel Hepatocyte Model, HepatoPac". (2013). *Drug Metabolism and Disposition 41*, 2024-2032. doi:10.1124/dmd.113.053397
http://dx.doi.org/10.1124/dmd.113.053397

Appendix A: Study Checklist

It is helpful to answer the following questions before beginning a Metabolic Stability study with HEPATOPAC cultures. Use this as a guide to help complete the Metabolic Stability Dosing Plate Map (Appendix B) and the Metabolic Stability Sample Collection Block Map (Appendix C).

1. What is the number of compounds that will be tested?
2. How many time points will be taken for each?
3. How many replicates will there be per time point?
4. What concentrations of the compound will be evaluated? (The standard is 1µM)
5. What solvents, besides DSMO will be needed for the compounds being evaluated?
6. Which controls will be used (See Table 3)?

Appendix B: Metabolic Stability Dosing Plate Map

For each well, record compound name or identification code, replicate number, time dosed, and concentration.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Cpd 1	Cpd 1	Cpd 1	Cpd 1	Cpd 1	Cpd 1	Cpd 3	Cpd 3	Cpd 3	Cpd 3	Cpd 3	Cpd 3
C	Cpd 1	Cpd 1	Cpd 1	Cpd 1	Cpd 1	Cpd 1	Cpd 3	Cpd 3	Cpd 3	Cpd 3	Cpd 3	Cpd 3
D	Cpd 1	Cpd 1	Cpd 1	Cpd 1	Cpd 1	Cpd 1	Cpd 3	Cpd 3	Cpd 3	Cpd 3	Cpd 3	Cpd 3
E	Cpd 2	Cpd 2	Cpd 2	Cpd 2	Cpd 2	Cpd 2	Cntrl	Cntrl	Cntrl	Cntrl	Cntrl	Cntrl
F	Cpd 2	Cpd 2	Cpd 2	Cpd 2	Cpd 2	Cpd 2	Cntrl	Cntrl	Cntrl	Cntrl	Cntrl	Cntrl
G	Cpd 2	Cpd 2	Cpd 2	Cpd 2	Cpd 2	Cpd 2	Cntrl	Cntrl	Cntrl	Cntrl	Cntrl	Cntrl
H												
Time Point (Hours)	**0**	**4**	**24**	**48**	**96**	**168**	**168**	**96**	**48**	**24**	**4**	**0**

Ensure that only the 0-hour time point is dosed in columns 1 and 12 to prevent exposure of later time points to edge well evaporation.

Appendix C: Metabolic Stability Sample Collection Block Plate Map

For each well, record compound name or identification code, replicate number, time dosed, and concentration.

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B1											
C1											
D1											
E1											
F1											
G1											
H1											