HEPATOPAC® Kit

Metabolite Identification Protocol

Use of HEPATOPAC for the Identification of Compound Metabolites

TP-003 V1.0

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Product Description

This instruction manual describes the use of HEPATOPAC Kits to conduct Metabolite Identification (MetID) studies.

> NOTE:
> Read these instructions in their entirety before starting to unpack boxes 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
- 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 Conical Tubes and 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
- Deep well (2mL) blocks or equivalent, for preparation of dosing solutions

Handling/Caution Statement

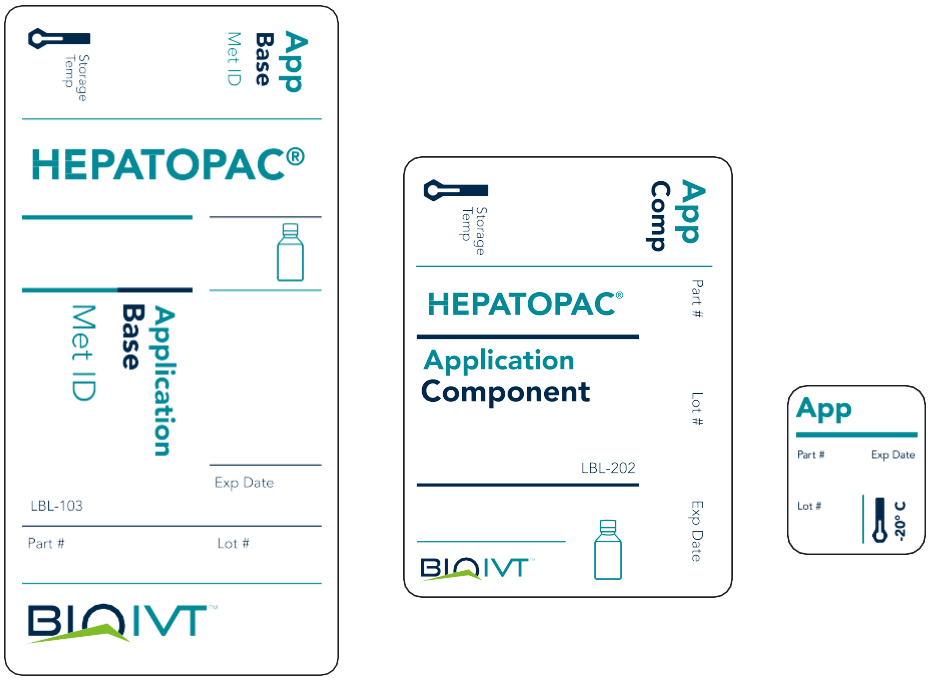
1. Do not use a vacuum-powered aspiration device with HEPATOPAC plates.
2. Avoid scraping the monolayer with pipette tips. This may cause damage to the monolayer in that region of the well.

Protocol

NOTE:
Prior to initiating a MetID study using HEPATOPAC, copy and fill out the Study Checklist (Appendix A) and Dosing Plate Map (Appendix B).

Metabolite Identification: Application Medium Preparation

1. Locate the bottles labeled as follows:
   - Application Base MetID
   - 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 # 5010C 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 MetID Bottle(s). Note the following:

- Refer to the appropriate column in Table 1 for the volume of each component required for either the 125 mL bottle, 250 mL bottle or the 500mL 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/have been reconstituted with the components, it is referred to (in this protocol) as "**MetID 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 MetID Application Medium.
The final volume in the 250 mL bottle will be 250 mL of MetID Application Medium.
The final volume in the 500 mL bottle will be 500 mL of MetID 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
5010C	3.6 mL	9 mL	18 mL
5011C	1 µL	2.5 µL	5.0 µL
5012C	1 µL	2.5 µL	5.0 µL

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

Metabolite Identification: Study Initiation

NOTE:
Initiate the MetID 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.

1. Warm the MetID Application Medium (containing appropriate components as described above) 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 in the BSC:
    - Reagent Reservoirs
    - Pipette Aid
    - Serological Pipettes (10-25mL)
    - 50-mL Conical Tubes and Rack
    - Sterile Pipette Tips
    - Pipette

- Pre-warmed Met ID Application Medium

NOTE:
BSL-2 practices must be followed during all work with HEPATOPAC. 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.

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.

5. Carefully place the HEPATOPAC plates in the BSC.

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 MetID Application Medium:
   - To wash: Remove the maintenance medium and apply the appropriate species-specific volume of MetID Application Medium to each well. Refer to Table 2 below for species-specific volumes.
   - Remove the MetID Application Medium and re-fill the wells with the appropriate species-specific volume of fresh MetID Application Medium.

Table 2: Species-specific Volume for Protocol

Human	Rat	Monkey	Dog
400µL	300µL	400µL	400µL

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, 10% 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 in the BSC:
   - Sterile DMSO
   - Compounds
   - 1-2 mL 96-well Deep Well Sample Collection 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. 10 mM). Refer to Table 3 for reference compound information.
   - The standard MetID dosing concentration is 10µM.

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

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

$$Volume = Mass \div (Molecular\ Weight * Molarity)$$

Table 3: MetID Reference Compound Information

Parent Compound	Expected Metabolites	4 Hour	2 Days	7 Days
Ziprazidone "Z"	Z. Parent Compound (Ziprasidone) 1. N-dealkylziprasidone S-oxide 2. ziprasidone S-oxide 3. S-methyldihydroziprasidone	1 2 3 Z	1 2 3	2 3
Linezolid "L"	L. Parent Compound (Linezolid) a. Morpholine ring-opened acid metabolite b. Morpholine ring-opened acid metabolite	a L	a L	a b L

3. In an appropriately sized conical tube, prepare a 2X dosing solution of each compound in MetID 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 Base Medium.

Table 4: Volume of 2x MetID Dosing Solution Required per Well

Human	Rat	Monkey	Dog
200 µL	150 µL	200 µL	200 µL

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.

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

4. Prepare a 2X solution of vehicle control by diluting the appropriate solvent in MetID Application Medium at the same percentage as used to prepare the 2X dosing solution of test compounds.
5. Aliquot the 2X dosing solutions of test compounds, control compounds, and vehicle control into deep well blocks for efficient dosing.

Compound Dosing

NOTE:
Only work with two plates at a time to prevent the 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 MetID Application Medium to each well at half the final dosing volume (see Table 5).
2. Apply 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
Met ID Application Medium	200 µL	150 µL	200 µL	200 µL
2X Dosing Solution	200 µL	150 µL	200 µL	200 µL
Final Dosing Volume (1X)	400 µL	300 µL	400 µL	400 µL

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

NOTE:
Depending on the study design, a t=0 sample may be collected from a dosed well or prepared from the 2X dosing stocks and diluted to 1X in equal volume of fresh MetID Application Medium.

3. Gently swirl the plates and return them to the 37°C, 10% CO_2 incubator.
4. Record start time.

5. Monitor culture morphology throughout the application. Document morphology of cultures with phase contrast images.

Metabolite Identification: Sample Collection

NOTE:
Refer to the Sample Collection Block Plate Map (Appendix C) prior to beginning sample collection.

The following protocol is for collection of culture supernatants. Depending on 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 MetID 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 Met ID Application Medium
    - Sample collection supplies, i.e. 1-2 mL Deep Well Blocks, Cap Mats, Sealing Press
    - Dry Ice
    - Timer
3. Properly label the collection block and place on dry ice. Record the time of sample collection for each time point.
4. At each time point, collect the entire sample supernatant and place in the sample collection plate.
5. Replace the collected supernatant with the appropriate species-specific volume of fresh MetID 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.

Appendix A: Study Checklist

It is helpful to answer the following questions before beginning a MetID study with HEPATOPAC cultures. Use this as a guide to help complete the MetID Dosing Map (Appendix B) and the MetID 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? (The standard is 0 hour, 4 hour, 2 days, 7 days)
3. How many replicates will there be per time point?
4. What concentrations of the compound will evaluated? (The standard is 10µM.)
5. What solvents, besides DSMO will be needed for the compounds being evaluated?
6. Which controls will be used? (Include functional controls, compound comparison controls, etc.) NOTE: The standard controls are Ziprasidone and Linezolid (see Table 3).

Appendix B: MetID Dosing Plate Map

For each well, record compound name or identification code, replicate number, time dosed, and concentration (if other than 10µM).

A1	A2	A3	A4	A5	A6
B1					
C1					
D1					

Appendix C: MetID Sample Collection Block Plate Map

For each well, record compound name or identification code, replicate number, time dosed, and concentration (if other than 10μM).

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