HEPATOPAC® Kit

Toxicity Protocol

Use of HEPATOPAC Cultures for Determination of Toxicity via ATP, ALT, GSH and Urea

TP-006 V1.0

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Product Description

This instruction manual describes use of HEPATOPAC Kits to conduct toxicity 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. (Note: Stromal only controls are required for the ATP and GSH assays. To determine the hepatocyte-specific effects of test compound(s), ATP/GSH levels determined from stromal-only cultures are subtracted from HEPATOPAC ATP/GSH values.)

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 Sterile Conical Tubes and appropriately sized Rack
- Sterile Reagent Reservoirs
- Sample Collection Supplies, i.e. 96-well Sample Collection Plates
- Deep Well Blocks
- Test Compound(s)
- Sterile DMSO (or other solvent for making compound stocks)

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Handling/Caution Statement

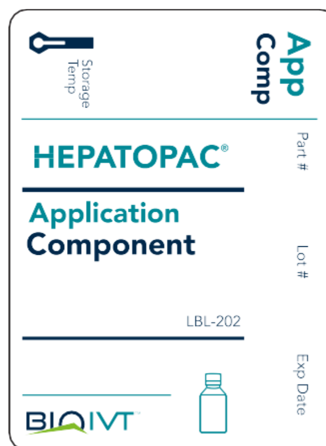
1. Do not use a vacuum-powered aspiration device with HEPATOPAC plates.
2. Avoid scraping the monolayer with pipette tips. This may cause damage to the monolayer in that region of the well.

NOTE:
Prior to initiating a Toxicity study using HEPATOPAC cultures, complete the Study Checklist (Appendix A).

Protocol

Toxicity Application: Medium Preparation

1. Locate the bottles labeled as follows:
   - Application Base Tox
   - 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 Tox Bottle(s). Note the following:

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- 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/have been reconstituted with the components, it is referred to (in this protocol) as "**Toxicity Application Medium**". Label the bottle 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 Toxicity Application Medium.
The final volume in the 250 mL bottle will be 250 mL of Toxicity Stability Application Medium.
The final volume in the 500 mL bottle will be 500 mL of Toxicity 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 Toxicity Application Medium to the appropriate storage conditions. (Refer to Step 3 above.)

Toxicity: Study Initiation

NOTE:
Initiate the Toxicity 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.

Follow the procedures listed below for HEPATOPAC plates when treating stromal-only control plates. Work with the stromal-only plates prior to handling HEPATOPAC plates.

1. Warm the Toxicity Application Medium (containing appropriate components as described in the section above entitled "Toxicity 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

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- 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.

These practices help to protect the user and maintain sterile conditions for HEPATOPAC cultures.

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

Table 2: Species-specific Volumes per Well

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

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 them in the BSC:
   - Sterile DMSO
   - Compounds
   - 1-2mL 96-well Deep Well blocks

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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. Prepare enough 200-1000X for the duration of the study. Refer to Table 3 for reference compound information. Refer to Table 4 for the required dosing volumes.

> 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

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

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

3. Serially dilute (1:2) the 200-1000X DMSO stock of the top concentration in DMSO to create 7 stocks at 200-1000X of the final test concentrations. On each study dosing day, use 200-1000X stocks of the top concentration to make fresh serially diluted stocks.
4. Use 200-1000X DMSO stocks to prepare 2X Dosing Solutions of each of the 7 concentrations in Toxicity Application Medium. For example, to make 2X stock from a 1000X stock, add 2µL of 1000X compound stock to 998µL of Toxicity Application Medium.
5. Prepare a 2X solution of vehicle control (0µM) by diluting the appropriate solvent in Toxicity Application Medium at the same percentage as used to prepare the 2X dosing solution of test compounds.
6. Mix well.

> 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.

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Table 3: Toxicity Reference Compound Information

Compound	DILI Classification	Tested Species	C_{max}[1]	Suggested Concentrations
Aspirin	DILI Negative	Human	5.5 µM	100, 50, 25, 12.5, 6.25 ,3.125, 1.5625, 0 C_{max}
Amiodarone	DILI Positive	Human	0.8 µM	100, 50, 25, 12.5, 6.25 ,3.125, 1.5625, 0 C_{max}

Compound Dosing

NOTE:
Only work with two plates at a time to prevent cultures from being held in minimal medium 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 Toxicity Application Medium to each well at half the final dosing volume (see Table 4).
2. Apply an equal volume (see Table 4) of 2X dosing solution(s) to each well.

Table 4: Dosing Volumes per Well

Medium Type	Human	Rat	Monkey	Dog
Toxicity 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

3. Gently swirl the plates and return them to the 37°C, 10% CO_2 incubator.
4. Monitor culture morphology throughout the application. Document morphology of cultures with phase contrast images.

Toxicity: Sample Collection and Re-Dosing

1. Warm the Toxicity 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 Toxicity Application Medium
    - Sample collection supplies, i.e. 1-2 mL Deep Well Blocks, Cap Mats, Sealing Press
3. Properly label the collection plate. Reference Appendix B for the plate template.
4. At each time point:
    - Refer to Table 5 for the Example Dosing and Endpoint Schedule

[1] Xu, J., Henstock, P., Dunn, M., Smith, A., Chabot, J., and D. de Graaf. 2008. Cellular Imaging Predictions of Clinical Drug-Induced Liver Injury. TOXICOLOGICAL SCIENCES 105(1), 97-105.

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- Collect the entire volume of supernatant and place in the 96-well sample collection plate. Store supernatants appropriately according to your endpoint protocol.
  - (i) Store supernatants for Urea frozen at -80°C until they are ready to assay.
  - (ii) Do not freeze supernatants prior to running the ALT assay. The ALT endpoint assay must be run on the day samples are collected.
  - (iii) ATP samples much be run on the day of collection.
- Either re-dose the cultures or run a destructive endpoint assay. See Steps 5 and 6 below.

5. Time points requiring re-dosing:
   - Refer to Table 5 for Example Dosing and Endpoint Schedule.
   - After removal of sample supernatant, immediately apply fresh Toxicity Application Medium to each well at half the dosing volume. (See Table 4) **Wells should not be left dry**.
   - Apply equal volume of 2X dosing solution(s) to appropriate wells.
6. Time points requiring destructive endpoint assays:
   - Refer to Table 5 for Example Dosing and Endpoint Schedule
   - After removal of sample supernatant, immediately apply fresh Toxicity Application Medium to each well at the appropriate species-specific volume. (See Table 2) **Wells should not be left dry**.
7. Endpoint Protocols: Follow the assay protocol(s) of choice or contact BioIVT for suggested sample assay protocols.

Table 5: Example Dosing and Endpoint Schedule

Days Post Initial Dosing	0	1	2	3	4	5	6	7	8	9
Compound Dosing	✓		✓			✓		✓		
Non-Destructive Endpoints (Urea, ALT)			✓			✓		✓		✓
Destructive Endpoints (ATP, GSH)						✓				✓

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Appendix A: Study Checklist

It is helpful to answer the following questions before beginning a Toxicity study with HEPATOPAC cultures..

1. What is the number of compounds that will be tested?
2. How many time points wil 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 maximum test concentration is 100*C_{max})
5. What solvents, besides DSMO will be needed for the compounds being evaluated?
6. Which controls will be used?

Appendix B: Toxicity Sample Collection Block Plate Map

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

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

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