AccuLiver™

HUMAN BILIARY CLEARANCE KIT

Instruction manual to implement a biliary clearance assay using B-CLEAR® Technology

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About the ACCULIVER Human Biliary Clearance Kit

The ACCULIVER Hunan Biliary Clearance Kit, when combined with TRANSPORTER CERTIFIED™ human hepatocytes, is used to quantitate biliary clearance using B-CLEAR Technology.

See Appendices 1 and 2 for more information about TRANSPORTER CERTIFIED hepatocytes B-CLEAR Technology respectively.

The kit can be used for both disposition and interaction studies. The ACCULIVER Human Biliary Clearance Kit enables our clients to duplicate the experimental conditions in-house that we have developed and routinely use in our laboratory.

Limited License to Use B-CLEAR Technology

BioIVT grants the purchaser of the kit a limited license to use B-CLEAR Technology to conduct studies using the materials in the kit. By opening the sealed package, the purchaser and user of the kit agree to the terms of the license.

See Appendix 10 for the License Agreement.

Technical Assistance

Contact BioIVT Technical Support with questions about the ACCULIVER Biliary Clearance Kit, QUALGRO™ media, and this instruction manual.

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Product Description

The ACCULIVER Human Biliary Clearance Kit is designed to evaluate the hepatobiliary disposition (hepatic uptake, intracellular concentration, and biliary excretion) of test compounds. Two examples are provided: (1) Evaluation of the disposition of a test compound and (2) Evaluation of drug – drug interactions of two test compounds. Standard study designs are described below:

Hepatobiliary Disposition Study

A hepatobiliary disposition study is used to estimate hepatic uptake, intracellular concentration, hepatic efflux and biliary clearance of a test article(s). The kit allows various study design configurations; however, a standard design has the following parameters:

- Test Articles: 3
- Concentrations: 3
- Replications: 3
- Time Points: 1

Appendix 3 provides plate map diagram for the standard design described here.

The assay consists of triplicate parallel incubations of hepatocytes in "Plus (+) Buffer" and "Minus (-) Buffer"[1] with the test article. Each row of a 24-well plate consists of three Plus (+) wells and three Minus (-) wells. In 24-well format, four separate conditions (i.e. different test articles or multiple concentrations) may be assessed on a single plate.

Incubation times of 10 to 20 minutes are suitable for most test articles, and the study may be designed for multiple time points by using one plate per time point6. However, the assay is not compatible with incubation times that exceed 30 minutes. If more than one time point is included in the study design, all treatments are repeated at the other time point. Depending on the study design, the additional time point(s) may require additional kits.

It is important to consider the expected *in vivo* concentration, protein binding, blood-to-liver ratio, solubility, and analytical sensitivity in determining the test article concentration(s) for the study. Frequently studies are designed with 3 or more test article concentrations.

A system positive control must be included in one row of one 24-well plate. Taurocholate is recommended as a positive control for all disposition experiments. Deuterated or radiolabeled taurocholate can be used for the analysis.

A non-specific binding plate should be included in each study design. Biliary disposition is estimated by comparing test article, or its metabolites in the cell lysates from the (+) Buffer wells to test article, or its metabolites in cell lysates from the (-) Buffer wells.

[1] The "Plus Buffer" cause tight junctions between hepatocytes in the matrix to remain closed. The "Minus Buffer" causes them to open.

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Drug-Drug Interaction Study

A drug-drug interaction study is used to evaluate the effect (e.g. inhibition of hepatic uptake or efflux) of one test article on the hepatic disposition of another test article.

The kit allows various study design configurations; however, a standard design has the following parameters:

- Test Articles: 2
- Concentrations: 3
- Inhibitors: 1
- Replications: 3
- Time Points: 1

Appendix 4 provides plate map diagram for the standard design described here.

The assay consists of triplicate parallel incubations of hepatocytes in "Plus (+) Buffer" and "Minus (-) Buffer"[2] with one test article in the absence or presence of the other test article. Each row of a 24-well plate consists of three Plus (+) Buffer wells and three Minus (-) Buffer wells. In 24-well format, four separate conditions (i.e. different test articles or multiple concentrations) may be assessed on a single plate.

Each test article, at every concentration, is evaluated in both the absence and presence of an "inhibitor". Generally, the punitive inhibitor is pre-incubated in the hepatocytes (to allow for hepatic uptake of the punitive inhibitor), and then co-inhibited with the test article (to allow for direct effects on uptake). The pre-incubation time is in the 10-15 minute range to allow sufficient time for uptake and efflux. Longer exposure times (up to 30 minutes) may be useful if uptake or efflux is slow. The positive control (i.e. taurocholate) is also evaluated in the presence and absence of an inhibitor using the methodology described above.

Incubation times of 10 to 20 minutes are suitable for most test articles, and the study may be designed for multiple time points. However, the assay is not compatible with incubation times that exceed 30 minutes. If more than one time point is included in the study design, all treatments are repeated at the other time point. Depending on the study design, the additional time point(s) may require additional kits.

It is important to consider the expected *in vivo* concentration, protein binding, blood-to-liver ratio, solubility, and analytical sensitivity in determining the test article concentration(s) for the study. Frequently studies are designed with 3 or more test article concentrations.

[2] The "Plus Buffer" cause tight junctions between hepatocytes in the matrix to remain closed. The "Minus Buffer" causes them to open.

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A system positive control must be included in one row of one 24-well plate. Taurocholate is recommended as a positive control for all disposition experiments. Deuterated or radiolabeled taurocholate can be used for the analysis.

A non-specific binding plate should be included in each study design.
Biliary disposition is estimated by comparing test article, or its metabolites in the cell lysates from the Plus (+) Buffer wells to test article, or its metabolites in the cell lysates from the Minus (-) Buffer wells.

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Kit Contents

All materials in the kit must be stored at 4°C, except for the plates which may be stored at room temperature.

The ACCULIVER Human Biliary Clearance Kit contains the following items:

- TRANSPORTER CERTIFIED cryoplateable human hepatocytes (6 vials) (shipped separately)
- BD BioCoat 24-well plates, 5
- QUALGRO Thawing Medium, 250 mL
- QUALGRO™ Seeding Medium, 200mL
- QUALGRO™ Overlay Medium, 100mL
- QUALGRO™ Culture Medium, 250mL
- Plus (+) Buffer, 250mL
- Minus (-) Buffer, 250mL
- Antibiotic Mix, 10mL

Other Required Reagents, Materials and Equipment

To conduct the assay, it is necessary to acquire the following reagents, materials and equipment.[3]

Materials Shipped Separately

TRANSPORTER CERTIFIED™ cryopreserved human hepatocytes are shipped separately and must be stored in LN2 vapor.

Equipment and Materials

- Trypan Blue (Sigma, T8154)
- Matrigel™ (Corning, 354234)
- Biosafety Cabinet (BSL2)
- Humidified tissue culture incubator (37°C, 5% CO_2)
- Water bath (37°C)
- Slide Warmer (surface temperature set to 37°C)
- *Recommended:* 6-Channel EXP Impact2 Electronic Pipettor, 1250µL capacity (Matrix catalog # 2624), or equivalent**,** and compatible tips
- *Recommended:* 6-channel aspiration manifold (V.P. Scientific catalog # VP182C)
- *Recommended:* 6-channel partitioned reservoirs (FisherScientific AWLS-S30030)

[3] The experimental procedure detailed in this Kit assumes the end user is able to perform microplate reader assays.

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Study Preparation

Prepare Test Compound Stock Solution

Prior to beginning the experiment, stock solutions of all test compounds should be prepared. The general rule of thumb is to prepare a stock at 1000X the desired treatment concentration, and then use a 1:1000 dilution into medium to prepare dosing solutions. The most common solvent used for this is DMSO, although methanol and water are acceptable if required due to solubility constraints. For example, for a dose at 50µM, a 50mM stock should be prepared, and add 1µL stock for each mL of dosing solution, giving a solvent contribution of 0.1%. Sandwich-cultured human hepatocytes can tolerate up to the addition of 0.3% solvent content from test article stock solutions. It is best practice to maintain the same final (organic) solvent content across all treatments.

This kit can be used to assess the test articles across a broad range of concentrations, depending on cytotoxicity data and solubility limitations. BioIVT recommends that test articles be prepared at the following concentrations:

- C_{Max}
- 20X C_{Max}
- 50X C_{Max} or limit of solubility

Solubility Testing for Test Compounds

It is important to note that DMSO solubility may not be equivalent to aqueous (aka in cell culture media) solubility.

Prior to beginning the experiment, all new test compounds should be evaluated for solubility in Plus (+) Buffer and Minus (-) Buffer; extra media is provided for this purpose. Spike 1µL of stock solution into 1mL of warm **medium** and maintain at 37°C for the exposure interval. Any cloudiness or precipitation indicates incomplete solubility, and a lower concentration of test compound is required.

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Cell Culture Study Protocol

Day 0: Thaw and plate hepatocytes

Thawing Procedure:

1. Thaw and add 100x Antibiotic Mix at 1x final concentration to all media prior to use.
2. Remove vial(s) of cryopreserved hepatocytes from liquid nitrogen storage
3. Immediately suspend vial(s) up to the cap in a water bath set for 37°C
4. Incubate vials in water bath for 1.5 – 2 minutes until the vials are ~90% thawed. There should still be a small, visibly frozen portion remaining in the vial(s).
5. Immediately remove vial(s) from the water bath, wipe down with ethanol and transfer to the tissue culture hood.
6. Transfer the contents of the thawed vials (up to ~ $40x10^6$ cells total or 4 vials) into 45mL of pre-warmed (37°C) QUALGRO Thawing Medium in a 50mL conical tube. The QUALGRO Thawing Medium does contain percoll so an additional purification step is not required.
7. Rinse each vial 1 time by adding 1 mL of QUALGRO Thawing Medium from the conical tube to each vial and decanting the volume back into the 50mL conical tube.
8. Gently invert 50mL conical tube 3-5 times to mix.
9. Centrifuge at 100 x g for 8 minutes to pellet the cells.
10. Aspirate QUALGRO Thawing Medium supernatant.
11. Determine live cell yield and viability (Appendix 5).

See Appendix 11 for example pictures of hepatocytes in culture that have formed a proper matrix.

Plating

12. Dilute cell suspension with warm (37°C) QUALGRO Seeding Medium to the final concentration specified on the specific lot's TRANSPORTER CERTIFIED hepatocyte Certificate of Analysis. (Plating density can vary from lot to lot and is specified on the BioIVT data sheet.)
13. Gently agitate cell suspension to ensure uniform suspension of the hepatocytes.
14. Transfer 0.5mL of hepatocyte suspension to each well of a 24-well BD BioCoat plate.
15. After the suspension has been added to all the plates, place plates in a 37°C incubator and vigorously shake each plate in a north-south-east-west pattern ~10 times to evenly distribute the cells within each well.
16. Periodically agitate plates and if desired, examine plates under microscope to assess proper adherence. Proper adherence of cryopreserved hepatocytes takes approximately 2 – 4 hours from time of seeding.

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Day 1: Overlay Hepatocytes

Hepatocytes must be overlaid 18 to 24 hours after seeding, using the ***cold*** (4°C) QUALGRO Overlay Medium, included in the kit. The following steps describe the overlay procedure:

1. Thaw and add 100x Antibiotic Mix at 1x final concentration to the QUALGRO Overlay Medium prior to use. The shelf-life will be 7 days upon addition.
2. Dilute Matrigel in cold (4°C) QUALGRO Overlay Medium to a final concentration of 0.25 mg/mL.
3. Remove the plates from the incubator for overlay.
4. Agitate plates to dislodge dead and/or poorly attached cells from the monolayer.
5. Aspirate the medium containing the dead/dislodged cells.
6. Add 0.5mL of QUALGRO Overlay Medium (containing Matrigel) to each well of the plates.
7. Nonspecific Binding Plates can be created by adding QUALGRO Overlay Medium (containing Matrigel) to previously empty wells in 24-well BD BioCoat plates as needed.

Days 2-4: Feed and Image Cells

Feed

On days 2 – 4 feed the cells with fresh medium per the following procedure:

1. Thaw and add 100x Antibiotic Mix at 1x final concentration to the QUALGRO Culture Medium prior to use. The shelf-life will be 7 days upon addition.
2. Remove plates from the incubator for feeding.
3. Aspirate the medium from the wells.
4. Add 0.5mL of warm (37°C) QUALGRO-Culture Medium to each well of the plates.
5. Nonspecific Binding Plates should be fed daily to maintain similarities in Overlay thickness and daily medium exchange.

Image Cells (optional)

BioIVT recommends photographing the cells on every day of the study. Treatment groups can then be compared for signs of overt toxicity. Overt toxicity can impact data quality.

The morphology of the hepatocyte cultures should be compared to solvent controls for any morphological alterations (e.g., changes in cell shape, cytoplasmic alterations, accumulation of vacuoles suggestive of dilated organelles and lipid droplets) indicative of cytotoxicity (Tyson, 1987) (Guillouzo, 1997).

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Day 5: Dose Cells and Collect Lysate

The dosing process is different depending on whether a **disposition study** or **interaction study** is being conducted. The following table describes the procedure for a disposition study and interaction study. The columns describing steps in which there is no difference in the procedures for the disposition and interaction study designs, are combined in the table below. Steps for which there is a difference are shown in separate columns.

Steps	Disposition Study	Interaction Study
Prepare Buffer	Titrate Plus (+) and Minus (-) Buffers, included in the kit to pH 7.4 with NaOH or HCl within 24 hours of use. Store at room temperature.	
Prepare Dose Solution	Refer to Appendix 6 for analytical considerations with respect to dose concentration. 1. Prepare stock solution of Test Compound in DMSO, water, or ethanol. **Note:** The concentration of organic solvent in the Dose Solution should not exceed 0.3%. Generally, the concentration of stock solution should be prepared at 1,000 - 10,000X that of the final Dose Solution concentration. 2. Prepare Dose Solution by diluting appropriate quantity of Test Compound stock solution into Plus (+) Buffer. Note: solubility of Test Compound may need to be considered. For each Test Compound and concentration administered, prepare sufficient volume to allow for the following: • 1mL for dose concentration verification • 0.3mL per NSB blank well (× 3 wells) • 0.3mL per well (× 6 wells/row × number of rows) • **Note:** Retain 1mL of Dose Solution to verify initial dose concentration. 3. Prepare Dose Solution for taurocholate positive control (see Appendix 7) Aliquot Dose Solutions into desired reservoirs.	Prepare the dose solutions in the same manner as for a disposition study, however, the Putative Inhibitor will need to be prepared in Plus (+) and Minus (-) Buffer for the preincubation step. The dosing solution containing the Test Compound in the absence and presence of the Putative Inhibitor can be prepared in Plus (+) Buffer.
Workstation Set Up	1. Arrange the work the workstation such that the following components are within close proximity: Slide warmer (set to 37°C), Vacuum line(s), Water bath (set to 37°C), Ice tray/bucket, Incubator, Timers 2. Aliquot warm (37°C) Buffers into reservoirs for easy access during the procedure, either in water bath or on slide warmer. It is recommended to use a single divided reservoir to allow both buffers to be drawn into the pipet simultaneously. 3. Aliquot a separate volume of cold (4°C) Plus (+) Buffer into a deep-well, large volume reservoir, on ice. Place reservoirs containing warm (37°C) Dose Solutions in water bath.	

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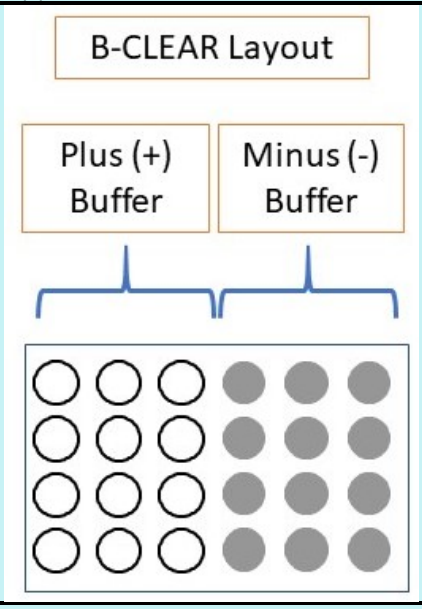
Steps	Disposition Study	Interaction Study
Plate Set Up	Set up the plates following the pattern show in Appendix 3. Assay Plates are conditioned with Plus (+) Buffer and Minus (-) Buffer, and then incubated with Test Compound or taurocholate (positive control). Each test condition (compound concentration and incubation time) is performed in triplicate pairs. Four separate Test Compounds or concentrations may be evaluated in a single assay plate, using rows A – D, respectively. The taurocholate positive control should be included in one row of one 24-well plate. Non-specific binding (NSB) wells are set up for each test article at each concentration, only in Plus (+) Buffer.	Set up the plates following the pattern shown in Appendix 4. Assay Plates are conditioned with Plus (+) Buffer and Minus (-) Buffer, in the absence and presence of the Putative Inhibitor (preincubation) and then incubated with Test Compound or taurocholate (positive control) in Plus (+) Buffer in the absence and presence of the Putative Inhibitor. Each test condition (compound concentration and incubation time) is performed in triplicate pairs. Four separate Test Compounds or concentrations may be evaluated in a single assay plate, using rows A – D, respectively. The taurocholate positive control should be included in one row of one 24-well plate without and inhibitor and one row with an inhibitor. Non-specific binding (NSB) wells are set up for each test article at each concentration, only in Plus (+) Buffer.
Assay Plates: Wash and Condition the Hepatocytes	1. Load 6-channel Matrix pipettor ("pipettor") with 1.25mL per channel warm Plus (+) Buffer and Minus (-) Buffers (3 channels of plus, 3 channels of minus). 2. Remove assay plate from the incubator & place on slide warmer. Using 6-channel aspiration manifold, aspirate media from all wells in the order indicated in Figure 4. 3. Dispense 0.3mL per well of *warm* Plus (+) Buffer and Minus (-) Buffer in the same order indicated in Figure 4. Swirl plate approximately 10 times while reloading pipettor with buffers. Aspirate buffer from wells in the same order. Repeat wash steps two more times. On the third wash, do not aspirate. Return plate to incubator for 10 minutes at 37°C. Start timer.	
Assay Plates: Incubate with Test Article	1. Remove plate from the incubator & place on slide warmer. Aspirate wells twice to remove all residual conditioning buffers. Dispense 0.3mL per well of *warm* Dose Solution into each column based on desired Plate Layout (e.g. Figure 4). Return plate to incubator. Stop and re-start timer. 2. Incubate for *desired time* at 37°C.	

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Steps	Disposition Study	Interaction Study
Wash the Hepatocytes	Load pipettor with 1.25mL per channel cold Plus (+) Buffer. Remove plate from the incubator but do not place on slide warmer. Aspirate wells twice to remove all residual Dose Solution. Note time on timer. Immediately dispense 0.3mL per well of cold Plus (+) Buffer into each well. Swirl plate for 20 seconds while reloading pipettor with cold Plus (+) Buffer. Aspirate buffer from wells. Repeat wash steps two more times. After third wash, aspirate twice to ensure that all residual buffer is removed. Note: It is important to note the time when the first 0.3mL is dispensed; 20 seconds from that time, start aspiration in the same order. This ensures consistent exposures across the entire plate	
Store Samples for Analysis	Seal plates with parafilm or in a vacuum-sealed bag. Freeze plates at -80°C for minimum of 15 minutes for later analysis.	

Sample Processing

The Assay Plates and NSB Control Plates must be stored at -80°C for at least 15 minutes, to facilitate lysis, prior to Sample Preparation. For a complete description of Sample Preparation prior to LC/MS analysis, see Appendix 6. Procedures specific for taurocholate positive control are described in Appendix 8.

Sample processing procedures specific for analysis of radiolabeled compounds are described in Appendix 9.

Data Analysis

Accumulation, biliary excretion index (*BEI*), and *in vitro* biliary clearance ($Cl_{biliary}$) are calculated for each Test Article. For the following calculations, all Cell Lysate and NSB values must be converted to a mass measurement (e.g., pmol per well) and normalized to protein content.

Calculate Accumulation and BEI

Use the following steps to calculate accumulation and biliary excretion index.

1. Correct for compound NSB using Equation 1

Subtract blank luminescence from all sample values per Equation 1.

Equation 1

$$Mass_{Sample} = Mass_{Uncorrected} - Mass_{NSB}$$

$Mass_{Sample}$ ≡ mass of Test Compound per well (corrected for NSB)

$Mass_{Uncorrected}$ ≡ mass of Test Compound per well (pmol)

$Mass_{NSB}$ ≡ mass of Test Compound nonspecifically bound per well (average of triplicate NSB lysate samples)

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2. Normalize to protein content using Equation

Use Equation 2 to normalize protein content

Equation 2

$$Accumulation_{Sample} = \frac{Mass_{Sample}}{\text{Protein}}$$

$Accumulation_{Sample}$ ≡ mass of Test Compound (corrected for NSB) per mg protein

Protein ≡ mg protein per well (refer to donor Transporter Certified Data Sheet or determine protein concentration using a protein assay according to your methods)

Note: The $Accumulation_{Sample}$ in the Minus (-) Buffer represents the amount of Test Compound inside the hepatocytes ($Accumulation_{Minus(-)}$). The $Accumulation_{Sample}$ in the Plus (+) Buffer represents the amount of Test Compound inside the hepatocytes plus bile pockets ($Accumulation_{Plus(+)}$).

Calculate BEI

Average the values of triplicate wells for $Accumulation_{Plus(+)}$ and $Accumulation_{Minus(-)}$ samples and calculate *BEI* (expressed as a percentage) using Equation 3:

3. BEI Equation

$$BEI = \frac{Accumulation_{Plus\,(+)} - Accumulation_{Minus\,(-)}}{Accumulation_{Plus\,(+)}} \times 100\,.$$

Calculate in vitro Biliary Clearance (CIbiliary)

Use the following steps to calculate *in vitro* Biliary Clearance ($Cl_{biliary}$).

Determine CIbiliary using Equation

$$Cl_{biliary} = \frac{Accumulation_{\text{Plus }(+)} - Accumulation_{Minus\ (-)}}{AUC} \qquad (9.2.1a)$$

$$AUC = DoseConcentration_{\ (uM)} \times IncubationTime_{(\min)} \qquad (9.2.1b)$$

AUC ≡ 10000 pmol*min/mL, assuming a 1.0µM Dose Solution and a 10-minute incubation time.

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Scale to kilogram body weight using Equation

$$Scaling\ factor\ =\ 2962\ mg\ protein/kg\ liver\ weight\ *$$ (9.2.2a)

*Derived from published literature (Sohlenius-Sternbeck, 2006) (Davies, 1993) and internal studies at Qualyst, using Equation 9.2.2b:

$$Scaling\ \ factor = \frac{mg\ \ protein}{\#\ of\ \ hepatocytes} \times \frac{10^6\ cells}{g\ liver} \times \frac{g\ liver}{kg\ body\ weight}$$

(9.2.2b)

and the following parameters:
134 x 10^6 hepatocytes/g liver
25.7 g liver/kg body weight

Data Interpretation

Taurocholate positive control

- BEI (%): 60-90%
- Accumulation ≥ 20 pmol/mg protein
- CllV ≥ 20 mL/min/kg

Test Article

- BEI (%) <10% not statistically significant
- BEI (%) <15%Low biliary excretion potential
- BEI (%) =15-35% Moderate biliary excretion potential
- BEI (%) >35% High biliary excretion potential

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Appendix 1: TRANSPORTER CERTIFIED Hepatocytes

TRANSPORTER CERTIFIED is an industry standard that ensures hepatocytes have physiologic transporter function, metabolic competence and appropriate regulatory pathway functionality, under culture conditions we have defined. In sandwich culture, the intracellular concentrations (ICC) in TRANSPORTER CERTIFIED hepatocytes reflect physiologic conditions because the hepatocytes express functioning uptake and efflux transporters and develop bile pockets similar to bile canaliculi.

The evaluation process for includes the following activities:

- Assessment of viability and phase II activity
- Quantitation of transporter activity using drug reference probes to assess uptake, biliary efflux, and basolateral efflux
- Assessment of the inhibition of these transporters as well as the intracellular concentrations of these same probes

Data from our assessment of a new lot is compared to our extensive historical database from fresh primary hepatocyte lots.

Appendix 2: B-CLEAR Technology

B-CLEAR technology is a patented methodology that allows quantitation of biliary efflux in an in vitro model using hepatocytes that form bile pockets that are analogous to bile canaliculi and have functioning uptake and efflux transporters. The bile pockets can be opened or closed, using B-CLEAR technology. This enables measurement of biliary efflux making it possible to estimate biliary clearance and evaluate biliary transporter interactions and conduct cellular mass balance measurements.

Data from models using B-CLEAR technology is used to predict clinically-relevant drug interactions, hepatotoxicity, transporter interactions and to support regulatory submissions.

We offer hepatic clearance research services using B-CLEAR Technology. Alternatively, Human Biliary Clearance kits and Rat Biliary Clearance kits, using B-CLEAR Technology are available to clients who prefer to conduct hepatic clearance studies in their own laboratories.

B-CLEAR Technology is covered by US Patent 6,780,580 and other worldwide patents issued & pending.

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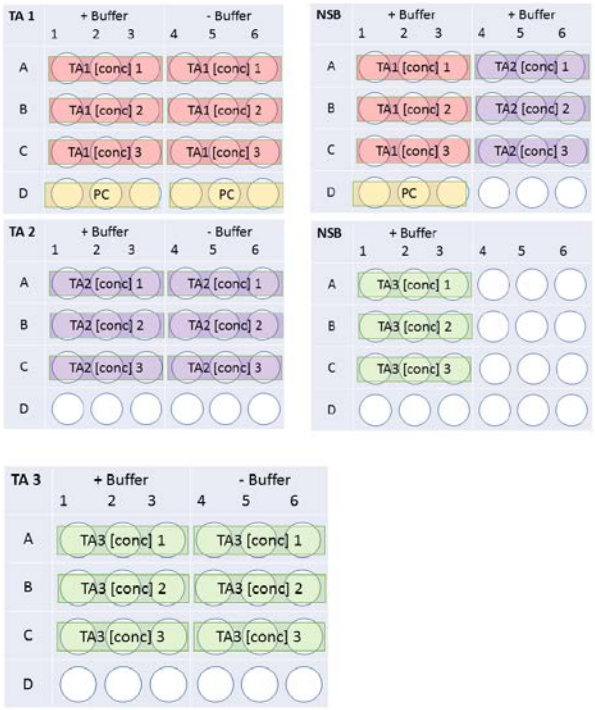
Appendix 3: Plate Map: Disposition Study

The kit allows various study design configurations. This appendix describes a design with the following parameters:

- Test Articles (TA): 3
- Concentrations [Conc]: 3
- Replications: 3
- Time Points: 1

Note the following critical factors in the study design:

- Each plate is set up with a + Buffer and – Buffer side, except for non-specific binding (NSB) plates, which only include + Buffer.
- Each TA at each [Conc] is included in an NSB plate.
- The positive control (PC) only needs to be included once in + Buffer and – Buffer, and in a NSB plate.
- Plates are preconditioned in Plus (+) and Minus (-) Buffer, and dosed with Test Compound in Plus (+) Buffer.
- The different TA concentrations are arranged in rows on the plate.



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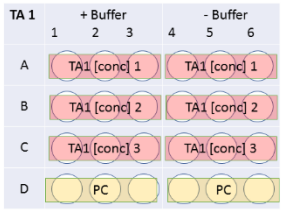
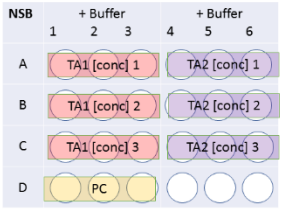
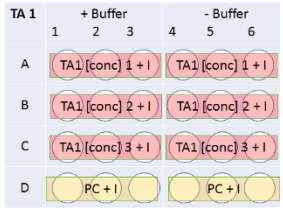
Appendix 4: Plate Map: Interaction Study

The kit allows various study design configurations. This appendix describes a design with the following parameters:

- Test Articles (TA): 3
- Concentrations [Conc]: 3
- Inhibitors (I): 1
- Replications: 3
- Time Points: 1

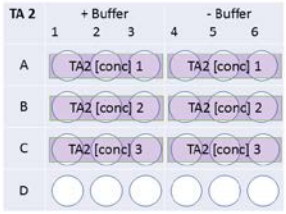


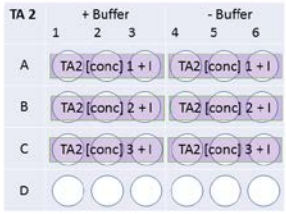




Note the following critical factors in the study design:

- Each plate is set up with a + Buffer and – Buffer side, except for the non-specific binding (NSB) plate, which only includes + buffer.
- Each TA at each [Conc] is included in the NSB plate.
- The positive control (PC) only needs to be included once in + Buffer and – Buffer, and in the NSB plate.
- Plates are preincubated in Plus (+) and Minus (-) Buffer, in the absence and presence of the Putative Inhibitor, and dosed with Test Compound in the absence and presence of the Putative Inhibitor in Plus (+) Buffer.
- The different TA concentrations are arranged in columns on the plate.





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Appendix 5: Determining Live Cell Yield and Viability

1. Remove 50µL of cell suspension and place into a clean microcentrifuge tube.
2. Add 400µL of the Seeding Medium
3. Add 50µL of Trypan Blue (Sigma, T8154) and mix by gently inverting tube several times (dilution factor = 10).
4. Place a clean cover slip over the chamber of the hemocytometer.
5. Fill one or both sides of the chambers with 10µL of cell suspension containing Trypan Blue. View under a microscope using 10X magnification. (Example of four squares being counted)
6. Count the number of viable cells (seen as bright cells) and non-viable cells (stained blue).
7. Calculate % viability:

$$[Percentage\ of\ Viable\ Cells] = \frac{[Total\ Number\ of\ Viable\ Cells]}{[Total\ Number\ of\ Cells]} \times 100$$

8. Calculate the number of cells/mL:

$$[Number\ of\ Cells\ /mL] = \frac{[Total\ Number\ of\ Viable\ Cells]x[dilution\ factor]x10^4}{Number\ of\ Squares\ Counted}$$

9. Calculate total number of cells:

$$[Total\ Number\ of\ Cells] = [Cells\ per\ mL]x[Total\ Liquid\ Volume]$$

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Appendix 6: Test Compound Sample Preparation for LC/MS

There are two matrices to consider prior to bioanalysis of disposition samples: 1) Lysate and NSB samples and, 2) Dose samples.
The sample preparation methods presented below are taken from previously performed experiments that have been applied successfully to a wide range of test compounds. The guidelines can be followed step-by-step or used as a general guide. For detailed material descriptions, sample preparation methods and LC/MS/MS analysis descriptions of a specific compound (d_8-TCA), see Appendix 3.

Note: 24-well Assay, NSB and Analytical Plates must be stored at -80°C for at least 15 minutes prior to sample preparation to facilitate cell lysis.

Cell Lysate Sample Preparation

1. To each well of a previously frozen 24-well plate, add 500mL 70:30 methanol:water, containing internal standard, and replace lid.
2. On a plate shaker (e.g., Lab-Line Instruments Model 4625) mix plate at speed setting of 3 (250 rpm) for approximately 15 minutes.
3. Transfer cell lysate to a Whatman® 96-well Unifilter® Plate with 25µM MBPP over 0.45µM PP (Catalog # 7770-0062). This filter plate is stacked on a Greiner 96-well Deepwell Plate (Bellco Catalog # 450-780270).
4. Centrifuge filter plate plus collection plate at 2000 rcf for 5 minutes.
5. Evaporate sample in collection plate to dryness under a flowing stream of nitrogen.
6. Reconstitute each sample with 200 mL of an LC/MS-compatible solution (mobile phase).
7. Shake plate for approximately 15 minutes.
8. Transfer reconstituted sample to a Whatman® 96-well Unifilter® Plate with 0.45µM PVDF (Catalog # 7700-2806). This filter plate is stacked on a Costar 96-well Plate (Catalog # 3956).
9. Centrifuge filter plate plus autosampler plate at 2000 rcf for 5 minutes.
10. Seal plate with a silicone capmat. Samples are ready for LC/MS analysis.

Dose Sample Preparation

1. Stack a Whatman® 96-well Unifilter® Plate with 0.45µM PVDF (Catalog # 7700-2806) on an autosampler Costar 96-well Plate (Catalog # 3956).
2. Transfer a small volume (10-20mL) of sample and a larger volume of LC/MS-compatible diluent (200-400mL, containing internal standard) to the Whatman® 96-well Unifilter® Plate.
3. Centrifuge filter plate plus autosampler plate at 2000 rcf for 5 minutes.
4. Seal plate with a silicone capmat. Mix. Samples are ready for LC/MS analysis.

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Preparation of Analytical Calibration Curves

Lysate and NSB Calibration Curves

It is recommended that 24-well Analytical Plates be generated as a matrix for preparing calibration curves to calculate the concentrations of test compound in Lysate and NSB samples. Analytical Plates are generated by taking Day 5 assay plates and washing & freezing, without any further incubations. Analytical Plates can be used to prepare quality control (QC) samples or, during method development, to test the parameters of the sample preparation and bioanalysis procedures.

Typically, a single row of a 24-well Analytical Plate is used for constructing a calibration curve. Five wells are used to prepare a 5-point calibration curve with the sixth well as a blank, to monitor potential carryover and background interferences. Data are expressed as total mass/well (e.g., pmol/well). If the range of the calibration curve is between 0.1% and 20% of the total dose, the range should be sufficient. (See Section 5.1.2 for equations estimating the low and high limits of the calibration curve.)
If the samples will be analyzed by LC/MS, the different levels of the calibration curve can be spiked with 10µL aliquots of spiking solutions at the appropriate concentrations.

Dose Sample Calibration Curves

Dose sample spiking solutions can be prepared in 1.8mL microcentrifuge tubes at the appropriate concentrations and in a matrix approximating the uptake matrix (e.g., Plus (+) buffer). If the range of the calibration curve for Dose Solution samples lies between 20% and 100% of the total dose, the calibration curve range should be sufficient.

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Appendix 7: Control Probes

Sodium taurocholate, an endogenous bile salt, is rapidly taken up by hepatocytes and extensively excreted into bile. Taurocholate is the suggested control probe for disposition studies. Either LC/MS/MS or liquid scintillation counting can be used to analyze the samples.

LC/MS/MS

A stable isotope of taurocholate, d_8-taurocholate, is used for dosing the cells and for creating the calibration curves for LC/MS/MS analysis. The bile acid, d_8-taurocholic acid (d_8-TCA), is detected by LC/MS/MS. d_8-TCA is analytically-distinguishable from endogenous taurocholic acid.

Preparation of d_8-taurocholate

Add 100µL DMSO to supplied vial of 0.683mg d_8-sodium taurocholate to generate a 12.5mM stock solution. To prepare Dose Solution, dilute to 2.5µM in an appropriate volume of Plus (+) Buffer. A minimum of 5mL of Dose Solution is required for one positive control row, three wells of an NSB plate, and Dose Solution verification.

Liquid Scintillation Counting

Sodium taurocholate and [^{3}H]-TCA are utilized for liquid scintillation counting.

Sodium taurocholate	Sigma catalog # T4009
Taurocholic acid [^{3}H(G)]-	Perkin Elmer catalog # NET 322250UC

The Dose Solution is prepared by combining sodium taurocholate and adequate [^{3}H]-Taurocholic acid to ensure analytical sensitivity.

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Appendix 8: Bioanalysis of d_8-Taurocholic Acid

Sample Preparation of B-CLEAR®-HU 24-well Assay Plate Lysate

Equipment

- Titer Plate Shaker (Lab-Line Instruments Model 4625)
- Centrifuge suitable for 96-well plate formats

Materials

- Whatman® 96-well Unifilter® Plate with 25µM MBPP over 0.45µM PP (Catalog # 7770-0062)
- Greiner 96-well Deepwell Plate (Bellco Catalog # 450-780270)
- Whatman® 96-well Unifilter® Plate with 0.45µM PVDF (Catalog # 7700-2806)
- Costar 96-well Plate (Catalog # 3956)
- d_8-sodium taurocholate (Qualyst # C1001)
- d_4-taurocholic acid (d_4-TCA) (Toronto Research Chemical # T008850) internal standard (IS)

Solution and Volume Requirements:

Lysis Solution containing Internal Standard:	70:30 methanol:water containing 25nM d_4-taurocholic acid
Lysis Solution Volume:	500µL
Sample Diluent:	60:40 methanol:10mM ammonium acetate (native pH)
Sample Diluent Volume:	200µL

Standard Spiking Solutions:

Standard spiking solution: d_8-TCA in methanol (µM)	Spiking solution transfer volume (µL)	Total d_8-taurocholic acid transferred (pmol/well)
10	10	100
5.0	10	50
0.5	10	5
0.1	10	1.0
0.05	10	0.5

Procedure

1. Transfer standard spiking solutions to previously frozen 24-well Analytical Plate.
2. Add lysis solution to previously frozen study sample cell plates and to plates containing standards.
3. Shake all plates on the plate shaker at a speed setting of 3 (approximately 250 rpm) for approximately 15 minutes. Total contact time of the lysis solution with cells, prior to the filtration step, is approximately 20-30 minutes.
4. Transfer cell lysate solution to the Whatman® 96-well Unifilter® Plate with 25µM MBPP over 0.45µm PP which is stacked on top of the Greiner 96-well Deepwell Plate. Filter lysate into

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the deepwell plate using centrifugation at 2000 rcf for 5 minutes. Evaporate filtrate to dryness under nitrogen.

5. Reconstitute samples in sample diluent and mix for 15 minutes on the plate shaker with a speed setting of 6 (approximately 500 rpm).
6. Transfer reconstituted sample to a Whatman® 96-well Unifilter® Plate with 0.45µM PVDF which is stacked on top of the Costar 3956 plate. Filter reconstituted sample into a Costar 3956 plate using centrifugation at 2000 rcf for 5 minutes.
7. Seal Costar plate with a silicone capmat for LC/MS/MS analysis.

Background Information Supporting Sample Preparation Procedures

1. Non-specific binding of TCA to plastic surfaces can occur, particularly in solutions with low ionic strength and a high aqueous content.
2. In some preparations, after the final sample has been refrigerated (4°C) for 12-24 hours, a slight cloudiness has been observed. This sample cloudiness does not affect TCA concentration but may adversely affect HPLC column life.
3. Liquid handling throughout this procedure may be performed manually or by using a liquid-handling robot with custom-written programs.

LC/MS/MS Analysis of d8-Taurocholic Acid

Instrumentation:

Mass Spectrometer		**HPLC**		**Autosampler**	
Thermo Electron TSQ Quantum Discovery MAX		Shimadzu LC-10ADvp Pumps Shimadzu CTO-10Avp oven		Shimadzu HTc – 96-well	
Polarity:	negative	**Total Run Time:**	10 minutes	**Temp (°C):**	4
Source:	Ion Max ESI	**Column:**	Thermo Scientific 100x1.0mM Hypersil GOLD (C18) with matching guard and pre-column filter	**Rinse Solvent:**	50:50 methanol : water
Scan Type:	SRM	**Flow rate:**	50µL/min	**Rinse Volume:**	1500µL after aspiration
Ion Transfer Tube Temp (°C):	310	**Column Temp (°C):**	35	**Injection Volume:**	10µL
Spray Voltage:	3125	**Mobile Phase A:**	60% 0.5mM ammonium acetate (native pH):40% methanol		
Sheath Gas Pressure:	20	**Mobile Phase B:**	20% 0.5mM ammonium acetate (native pH):80% methanol		
Collision Energy Voltage:	57	**Isocratic/Gradient Profile**	Initial 40% B; 0.2 to 4 min ramp to 100%B; hold 2 min; step back to initial over 1 min		
Resolution:	Unit Q1(0.7)/Q3 (0.7)	**Post-Column Solvent addition**	10µL/min 100% methanol to MS 100% of time		

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Analyte(s) and Internal Standard(s):

Compound Name	AKA	Compound Source	Molecular Weight	Salt	Ret. Time (min)	Precursor m/z	Product m/z	Calibration Curve Range
d_8-Taurocholic acid	d_8-TCA	Custom: Qualyst/ Martrex	545.73	Na +	4 to 6	522	128	0.5-100 pmol/ well
d_4-Taurocholic acid	d_4-TCA (IS)	TRC # T008850	519.73	none	4 to 6	518	124	12.5 pmol/ well

Background Information Supporting LC/MS/MS procedures:

1. With this ionization source, TCA negative ion signal intensity is suppressed in the presence of ammonium acetate. Ammonium acetate at no higher than 0.5mM (native pH) is highly recommended. Ammonium acetate below 0.5mM will result in less chromatographic retention.
2. With this ionization source, TCA negative ion signal intensity is approximately 2X higher with methanol than with acetonitrile. Methanol as the mobile phase organic solvent is recommended.
3. A mobile phase gradient utilizing methanol facilitates regular column cleaning of any highly retentive biologically compounds that may be introduced onto the column from certain extracts of biological materials. Without such regular column cleaning, chromatographic retention may decrease over time. In circumstances where a column fouling problem does not exist, isocratic conditions are appropriate. HPLC column life is extended with using a pre-column filter and guard column.
4. With this ionization source, the use of micro-bore LC (column i.d. 1.0mm) yields significantly greater sensitivity per mole, on-column, compared to narrow-bore LC (column i.d. 2.1mm). This improved sensitivity is related to the reduced delivery of ammonium acetate to the source using the 50µL/min flow rate associated with a micro-bore application compared to a narrow-bore application using a flow rate of 200µL/min. Perhaps with other ESI source designs or in applications with less demanding sensitivity needs, the use of a narrow-bore system may be an option.
5. A 10µL injection is used to help meet lower limit of detection needs. Given other independent factors that either contribute to or reduce chromatographic band broadening, this large injection volume (for a microbore application) did not contribute significantly to additional band broadening, which might reduce sensitivity. An injection volume of less than 10µL is highly recommended if sensitivity needs can be met with existing mass spectrometry hardware.
6. To minimize fouling of the ESI probe sample tube over a prolonged analysis period, HPLC column effluent is directed to the ESI probe only over the period of run time between t = 2 to t = 8 minutes.
7. To minimize fouling of the ESI probe sample tube over a prolonged analysis period, particularly while column effluent is diverted to waste, a continuous flow of 100% methanol enters the probe via a post-column mixing tee.

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Appendix 9: Analysis of Radioactive Compounds

The sample preparation methods presented below are taken from previously performed experiments that have been applied successfully to a wide range of test compounds. The guidelines can be followed step-by-step or used as a general guide for the liquid scintillation analysis of radiolabeled Test Compounds and taurocholic acid positive control.

Note: 24-well Assay, NSB and Analytical Plates must be stored at -80°C for at least 15 minutes prior to sample preparation to facilitate cell lysis.

Materials Required (Not Supplied)

General Supplies

- Scintillation counter
- Scintillation cocktail
- Scintillation vials and caps
- Orbital plate shaker
- Vacuum pump and trap system (radioactive)
- Radiation waste containers

Cell Lysis Reagents

Phosphate buffered saline (PBS), tablet	Sigma cat. # P-4417
Triton X-100	Sigma cat. # 234729
Antifoam-A	Sigma cat. # A-6582
Whatman® Unifilter® plate filter	Whatman cat. # 7770-0062
Whatman® Uniplate™ collection plate	Whatman cat. # 7701-1750

Cell Lysis Solution (1X PBS/0.5% Triton X-100/0.005% Antifoam-A)

- Add 5 tablets PBS and 5mL Triton X-100 per 1 L final volume.
- Prepare 1% Antifoam-A stock solution in 1X PBS/0.5% Triton X-100 solution.
- Dilute 1% Antifoam-A stock solution 5:1000 in 1X PBS/Triton X-100 solution.
- Store a 4°C.

Analytical Considerations for Radiolabeled Compound

Equation A.5.1 can be used to estimate the total disintegrations per minute (DPMs) of radiolabeled compound per well ($DPM_{Estimate}$), assuming at least 0.5% uptake of the Test Compound into hepatocytes is achieved (Note: 0.5% uptake is an estimate derived from historical data and may not apply to all Test Compounds).

$$DPM_{Estimate} = Volume_{Dose} \times Concentration_{Dose} \times SpecificActivity_{TestCompound} \times 2.22e6 \times 0.005 \quad \text{(A.5.1)}$$

$DPM_{Estimate}$ ≡ DPM of Test Compound per well.
$Volume_{Dose}$ ≡ the volume (0.3mL) of Dosing Solution applied to cells.
$Concentration_{Dose}$ ≡ concentration (pmol/mL) of Test Compound in the Dose Solution.
$Specific\ Activity_{Test\ Compound}$ ≡ Specific Activity (µCi/pmol) of the Test Compound.

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2.22×10^6 ≡ conversion factor (DPM/µCi).

If the $DPM_{Estimate}$ is below the analytical limit of detection, the concentration of Test Compound in the Dose Solution can be adjusted to increase the amount of compound taken up into the hepatocytes, or the ratio of radiolabeled to non-radiolabeled compound can be increased to achieve higher DPM per well.

Sample Collection and Protein Determination

The following lysis procedure is recommended when using radiolabeled Test Compounds for liquid scintillation counting (LSC).

1. Warm frozen assay plate(s) to ambient temperature (at least 5 minutes).
2. Lyse cells with 0.5mL per well PBS/0.5% Triton X-100/0.005% Antifoam-A.
3. Place plate on orbital shaker for 10-20 minutes.
4. Transfer 0.5mL from each well to Whatman® Filter plate on top of a Whatman® Collection plate.
5. Centrifuge samples at approximately 2000 rcf for 5 minutes.
6. Collect Cell Lysate sample for LSC from supernatant without disturbing the cell debris pellet. Measure DPMs in liquid scintillation counter.
   Note: A portion of the Cell Lysate sample may be retained to determine protein concentration (see below).
7. Remaining supernatant can stored at -20°C.

Calculation of Accumulation for Radiolabeled Compound

Equation A.5.2 can be used to calculate the Accumulation in pmol/mg protein of a radiolabeled Test Compound.

$$Accumulation_{sample}[pmol/mg\ protein] = \frac{\left(\frac{DPM_{sample}}{2.22\times10^{6}[DPM/\mu Ci]}\right)}{Specific\ Activity[\mu Ci/pmol]} \times \left(\frac{Concentration_{radioactive} + Concentration_{non-radioactive}}{Concentration_{radioactive}}\right) \Big/ Protein Concentration[mg/mL] \times Volume_{sample}[mL]$$

(A.5.2)

DPM_{sample} ≡ the disintegrations per minute obtained from counting the lysate
$Volume_{sample}$ ≡ volume (mL) of cell lysate that is counted
Specific Activity ≡ activity (µCi/pmol) of the radiolabel
$Concentration_{radioactive}$ ≡ concentration of the radiolabeled compound in the dose solution
$Concentration_{non\text{-}radioactive}$ ≡ concentration of the non-radiolabeled compound in the dose solution
Protein Concentration ≡ measured protein concentration (mg/mL)
2.22×10^6 ≡ conversion factor (DPM/µCi)

The accumulation values determined from equation A.5.2 are applied to equations 9.1.3 and 9.2.1a to estimate the biliary excretion index (BEI) and the *in vitro* biliary clearance ($Cl_{biliary}$).

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Appendix 10: Limited-Use License for B-CLEAR® Technology

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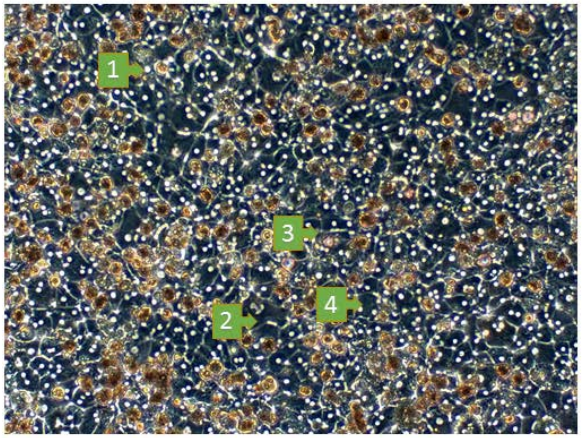
Appendix 11: Comparison of High Versus Low Quality Cultures of Hepatocytes

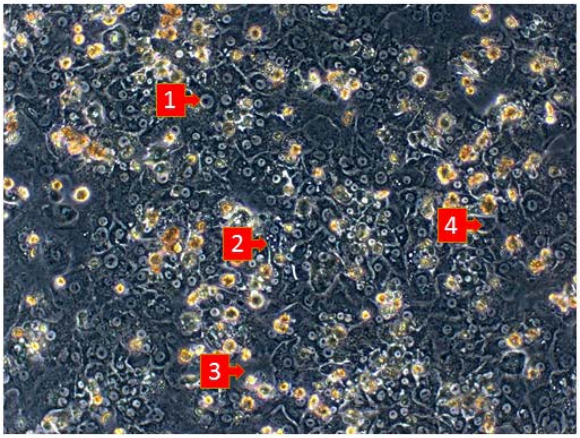
By Day 2 of culture, hepatocytes should begin to polarize and form bile pockets. Cells should be > 90% confluent and 100% confluent is ideal. Below is an example comparing good vs bad cultures of hepatocytes. Poor quality cultures should not be used in the study and should be discarded.

Hepatocyte Cultures

High Quality Culture
1. Clear, distinct nuclei
2. Clear cytoplasm: no swollen organelles; no intracellular debris.
3. Confluent monolayer: indicates tight cell-to-cell junctions, formation of bile canaliculi
4. Normal cell shape



Low Quality Culture
1. Indistinct nuclei
2. Swollen organelles and intracellular debris
3. Gaps in the monolayer; limited cell-to-cell interaction
4. Elongated, fibroblast-like cells



Caution: *This product was prepared from human tissue. Treat all products containing human-derived materials as potentially infectious, as no known test methods can offer assurance that products derived from human tissues will not transmit infectious agents.*

This product is being sold for research and/or manufacturing purposes only. The biological samples supplied by BioIVT, or any material isolated from the samples, are for in-vitro research use only and are not to be used as a source of material for clinical therapies. Human material may be used in vivo in animals. The user assumes all responsibility for its usage and disposal, in accordance with all regulations.

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