AccuLiver™

C-DILI KIT

Instruction manual to implement the cholestatic hepatotoxicity assay using TRANSPORTER CERTIFIED™ hepatocytes.

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Product Description

ACCULIVER™ Cholestatic Hepatotoxicity (C-DILI) Kits contain the materials needed to implement the C-DILI™ Assay, a novel *in vitro* method to evaluate a compound's potential for cholestatic drug-induced liver injury (DILI).

The C-DILI Assay has demonstrated high *in vitro – in vivo* correlation with compounds known to have a risk for cholestatic hepatotoxicity. The C-DILI Assay integrates multiple pathways critical in the development of cholestatic hepatoxicity, including BSEP inhibition, FXR antagonism, and inhibition of basolateral efflux transporters.[1] It is an effective tool for assessing the risk of cholestatic DILI and providing data useful in lead selection and managing toxicity risk.

ACCULIVER Cholestatic Hepatotoxicity (C-DILI) Kits enable users to duplicate the experimental conditions that we at BioIVT have developed and routinely use in our laboratory.

Materials and Storage

Upon receipt. Materials in the kit must be stored as stated below:

Kit Contents

- TRANSPORTER CERTIFIED cryoplateable human hepatocytes, 2 vials
- Collagen Coated 96-well plate, 1*
- QUALGRO™ Thaw Medium, 45 mL**
- QUALGRO™ Seeding Medium, 30 mL**
- QUALGRO™ Overlay Medium, 15 mL**
- QUALGRO™ Culture Medium, 30 mL**
- QUALGRO™ C-DILI Culture Medium, 30 mL**
- QUALGRO™ C-DILI Sensitization Medium, 30 mL **
- Supplement A, 400 µL *
- Supplement B, 40 µL ***
- C-DILI Negative Control, 20 µL ***
- C-DILI Positive Control, 20 µL ***
- Direct Toxicity Control, 20 µL ***
- Antibiotic Mix, 3 mL***

*Store at Ambient
**Store at 4°C
***Store at -20°C

Materials Shipped Separately

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

[1]Jackson, J.P., Freeman, K.M., St.Claire III, R.L., Black, C.B., and Brouwer, K.R. Cholestatic Drug Induced Liver Injury: A Function of Bile Salt Export Pump Inhibition and Farnesoid X Receptor Antagonism. APPLIED IN VITRO TOXICOLOGY, Volume 4, Number 3, 2018.

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Additional Required Equipment and Materials

The following reagents, materials and equipment which are not included in the kit are necessary to conduct the C-DILI Assay.[2]

Reagents

- Trypan Blue Solution, 0.4% (Gibco 15250061)
- Matrigel® (Corning 354234)
- DMSO (Sigma 34869)
- ATP Kit (Promega G7570)
- LDH Kit (Promega G7890)

Equipment and Materials

- 96-well deep well block[3]
- Hemocytometer
- Tissue culture hood (BSL2)
- Humidified tissue culture incubator (37°C, 5% CO2)
- Water bath (37°C)
- Vacuum pump and trap system
- Pipet devices and tips: 0-20 µL, 20-200 µL, 200-1000 µL
- Serological pipettor
- Sterile serological pipettes: 5 mL, 10 mL, 25 mL
- Conical centrifuge tubes: 15 mL, 50 mL
- Abs/Lum capable plate reader
- 96 Well Black Polystyrene Microplates (Corning Costar 3631)

[2] The experimental procedure detailed in this Kit assumes the end user is able to perform microplate reader assays.
[3] BioIVT recommends using a rubber cap mat for sealing, as aluminum or plastic foil seals will leak upon inversion.

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Handling/Caution Statement

All materials contained in the ACCULIVER Cholestatic Hepatotoxicity (C-DILI) Kit are considered safe to use for the purposes outlined in these instructions. Take care when handling any chemicals and use generally accepted good laboratory practices. Proper PPE should be worn while performing any cell culture assays.

Human hepatocytes (shipped separately) should also be treated with care when handling. While all BioIVT hepatocytes products are screened and are non-infectious, they should be handled utilizing universal precautions and in accordance with your institution's health and safety plan.

Protocol

Study Overview

The ACCULIVER Cholestatic Hepatotoxicity (C-DILI) Kit is designed to evaluate four (4) test articles, at three (3) concentrations, with three (3) replicates. Other study designs, such as EC_{50} determination, and evaluation of drug combinations are feasible with different configurations.

The kit contains sufficient media to culture TRANSPORTER CERTIFIED™ cryoplateable human hepatocytes in a single 96-well plate and will assess test articles in both standard QUALGRO culture medium and QUALGRO sensitization medium, in triplicate. The design includes positive controls for both direct (e.g. general) and cholestatic toxicity, one negative control, and one vehicle control. Cholestatic Hepatotoxicity is determined by identifying compounds which produce a hepatotoxic response only in sensitization medium.

TRANSPORTER CERTIFIED hepatocytes are cultured in sandwich culture format to reestablish physiologically-relevant uptake, metabolism, regulation and efflux function. The hepatocytes form a matrix with bile pockets and demonstrate transporter function, including function of NTCP, OATPs, BSEP, OSTs, and MRP3/4.

After the culture has been established, BioIVT's proprietary QUALGRO Sensitization Medium is added to half the wells, along with the test articles and controls, and incubated for 24 hours. The remaining half of the wells are incubated with test articles and controls prepared in standard QUALGRO C-DILI Culture Medium. All treatments including controls should be performed in triplicate. Hepatotoxicity is evaluated by measuring the release of lactate dehydrogenase (LDH) and depletion of ATP relative to controls in a standard plate-reader assay. Measurement of these cytotoxic endpoints in both medium allows the differentiation between compounds which are direct toxicants from those that are bile acid dependent (e.g. cholestatic) in their mechanism of toxicity.

The LDH response of the test compounds is compared to drugs with known clinical DILI effects. Comparison with the negative control, cyclosporine, and the positive controls, troglitazone for cholestatic toxicity, and the positive control, imatinib, for direct toxicity, allow compounds to be ranked for their clinical hepatotoxicity potential.[i] Cholestatic hepatotoxicity versus direct toxicity is determined by comparing treatments with QUALGRO C-DILI Sensitization Medium versus QUALGRO C-DILI Culture Medium.

Further experiments to elucidate the mechanism of cholestatic hepatotoxicity are recommended for any compounds which produce a positive result (i.e. a "hit").

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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. A general recommendation 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. For example, for a dose at 50 µM, a 50 mM stock should be prepared, and add 1 µL 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 0.25% 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 to account for higher portal vein concentrations compared to systemic concentrations:

- C_{Max}/C_{ss}
- 20X C_{Max}/C_{ss}
- 50X C_{Max}/C_{ss} 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 medium) solubility.

Prior to beginning the experiment, all new test compounds should be evaluated for solubility in QUALGRO C-DILI Culture Medium; extra medium is provided for this purpose. Spike 1 µL of stock solution into 1 mL of warm medium, and maintain at 37°C overnight. Any cloudiness or precipitation indicates incomplete solubility, and a lower concentration of test compound is required.

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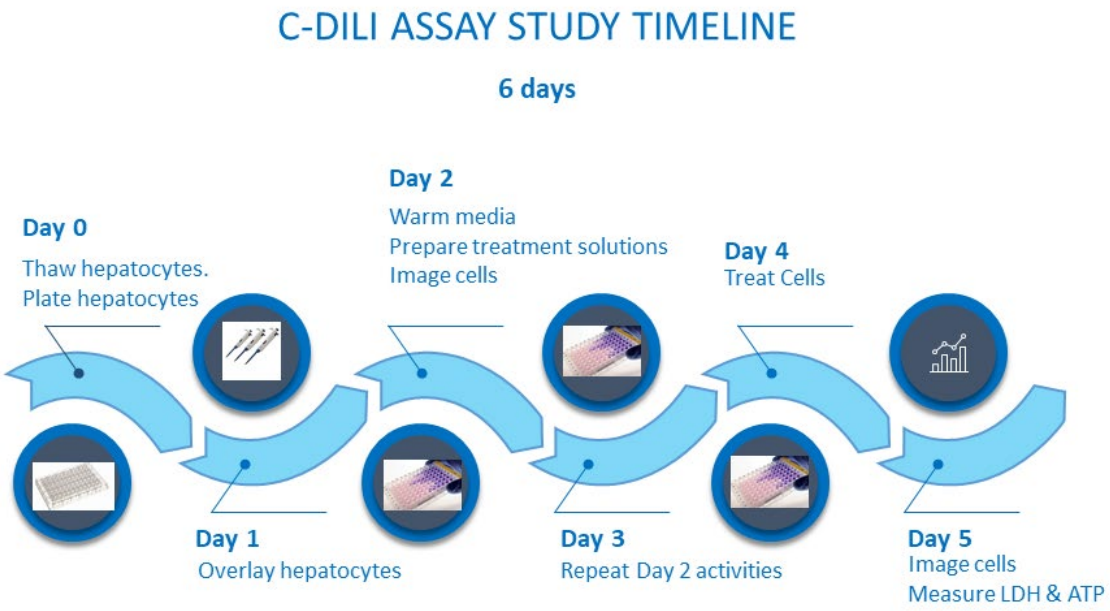
Cell Culture Study Protocol

The protocol for an experiment using the ACCULIVER C-DILI Assay Kit study is outlined in the diagram below.



Day 0: Thaw and Plate Hepatocytes

Note: The QUALGRO Seeding Medium should contain antibiotics. Thaw the Antibiotic Mix and add 10 µL to 990 µL medium prior to use. Upon adding the antibiotic mix, the QUALGRO Seeding Medium will have a 7-day shelf-life.

Thawing Procedure:

1. Remove vials of TRANSPORTER CERTIFIED cryoplateable human hepatocytes from liquid nitrogen storage.
2. Immediately suspend vials up to the cap in a water bath set for 37°C.
3. 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 vials.
4. Immediately remove vials from the water bath, wipe down with ethanol and transfer to the tissue culture hood.
5. Decant each vial (up to ~ 30 million cells total) into 45 ml warm (37^0 C) QUALGRO Thawing Medium in a 50 mL conical tube. QUALGRO Thawing Medium is included in the kit.

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6. Rinse each vial one time by adding 1 mL of QUALGRO Thawing Medium from the conical tube to the vial. Decant the material into the 50 mL conical tube. Gently invert 50 mL conical tube 3-5 times to mix.
7. Centrifuge at ~100xg for 8 min
8. Aspirate supernatant from tube, taking care not to disturb the cell pellet. Suspend the cell pellet with 1 mL warm (37°C) QUALGRO Seeding Medium, then add QUALGRO Seeding Medium up to 5 mL for every 1 million cells of expected yield (i.e., for a vial containing 5 million cells, resuspend cell pellet in 5 mL of QUALGRO Seeding Medium).

Seed Cells:

Determine viable cell yield (see Appendix 1), and 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.)

Pre-wet the 96-well collagen-coated plate with a 40 µL per well cushion of QUALGRO Seeding Medium. Gently agitate cell suspension to ensure uniform suspension of the hepatocytes. Using a reservoir and multichannel pipette, transfer 70 µL of hepatocyte suspension to each well of the plate.

Place the plate in a 37°C incubator but DO NOT SHAKE THE PLATE. Examine the plate under a microscope to assess proper adherence. Proper adherence of cryopreserved hepatocytes varies by lot and can take up to 24 hours.

See Appendix 2 for pictures of hepatocytes in culture that have formed a proper matrix.

Day 1: Overlay Hepatocytes

Hepatocytes must be overlaid 18 to 24 hours after seeding. Add cold (4°C) Matrigel to **cold** (4°C) QUALGRO™ Overlay Medium, included in the kit, at a final concentration of 0.25 mg/mL.

Note: The QUALGRO Overlay Medium should contain antibiotics. Thaw the Antibiotic Mix and add 10 µL to 990 µL medium prior to use. Upon adding the antibiotic mix, the QUALGRO Seeding Medium will have a 7 day shelf-life.

Place the completed overlay media and reservoir on ice; **DO NOT WARM** the QUALGRO Overlay Medium prior to application to cells. Once the cold QUALGRO Overlay Medium is added to the cells and the plate is returned to the incubator, the Matrigel within the QUALGRO Overlay medium will solidify into a gel, forming the "sandwich" around the hepatocytes. (Note: If the QUALGRO Overlay Medium is warmed prior to addition to cells, the entire contents will solidify, and the medium must be discarded.)

Remove the plate from the incubator and confirm cell attachment by microscopic examination. Once it is positioned in the biosafety cabinet, agitate the plate to dislodge dead and/or poorly attached cells from the monolayer, and aspirate the medium containing the dead/dislodged cells.

Using a reservoir and multichannel pipette, add 0.125 mL of **COLD** QUALGRO Overlay Medium supplemented with 0.25 mg/mL Matrigel to each well of the 96-well plate.

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Days 2 and 3: Image and Feed Cells

Warm Media

Warm the QUALGRO Culture Medium to 37°C.

Note: The QUALGRO Culture Medium should contain antibiotics. Thaw the Antibiotic Mix and add 10 µL to 990 µL medium prior to use. Upon adding the antibiotic mix, the QUALGRO Culture Medium will have a 7 day shelf-life.

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.

Cells may be photographed with or without media present, although some labs find it easier to take high-quality pictures without the medium. If medium is removed it should be returned immediately after the imaging is complete (<20 sec).

Feed Cells

Once positioned in the biosafety cabinet, aspirate the medium from the plate.

Using a reservoir and multichannel pipette, add 0.125 mL of Culture Medium to each well. Return plate to incubator.

Day 4: Image and Dose Cells

Warm Media

Warm the QUALGRO C-DILI Culture Medium and QUALGRO C-DILI Sensitization Medium to 37°C. Allow DMSO stock solutions to come to room temperature.

Note: The QUALGRO C-DILI Culture and Sensitization Medium should contain antibiotics. Thaw the Antibiotic Mix and add 10 µL to 990 µL medium prior to use. Thaw Supplement A and B and add 300 µL of Supplement A and 30 µL of Supplement B to the QUALGRO C-DILI Sensitization Medium.[4] Upon adding the antibiotic mix, the QUALGRO C-DILI Culture and C-DILI Sensitization Media will have a 7 day shelf-life.

Prepare Treatment Solutions

Treatment solutions should be prepared for all treatments prior to dosing the cells. Each test compound or control is prepared at 1 mL volume in both C-DILI™ Culture and Sensitization media. Assuming that test compound stock solutions are prepared at 1000X desired treatment concentration, treatment solutions are prepared by spiking 1 µL stock solution into 1 mL media[4].

See Appendix 3 for an example of a plate layout.

[4] Vortexing and gentle heating may be required to fully solvate.

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Treatment solutions should be prepared per the dilution scheme shown in Table 1.

Table 1: Treatment Solutions

Treatments	Stock Concentration	Treatment Concentration	Stock Volume	Media Volume
DMSO	100%	0.1%	1 µL	1 mL
Cyclosporin A	10 mM	10 µM	1 µL	1 mL
Troglitazone	75 mM	75 µM	1 µL	1 mL
Imatinib	40 mM	40 µM	1 µL	1 mL
Test Compound(s)	(1000X)	(1X)	1 µL	1 mL

Dose Cells

Maintain treatment solutions at 37°C (in incubator) until immediately prior to use.

Transfer the plate and the dosing block to the biosafety cabinet. Aspirate medium from the 96-well plate. Appendix 2 shows a plate map for a study design with 4 compounds, 3 concentrations, at 3 replicates. Study designs with a different combination of a compounds, concentrations and replications is possible.

Using a multichannel pipette, add 0.125 mL of treatment solution to appropriate wells.

Return the plate to incubator.

Day 5: Collect Data

Image Cells (Optional)

BioIVT recommends that visual examinations are performed for each treatment group and media combination, and any observations recorded. Do not discard media if imaging is performed on Day 5.

The morphology of the hepatocyte cultures should be noted 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).

Perform ATP and LDH Assays

The C-DILI Assay follows Promega's multiplexing protocol[2] for ATP (CellTiter-Glo® Luminescent Cell Viability Assay, Cat. No. G7570) and LDH (CytoTox-ONE™ Homogeneous Membrane Integrity Assay, Cat. No. G7890) assays. A brief summary protocol is provided as follows, but BioIVT recommends reviewing the respective Promega technical bulletins in detail prior to the experiment. Protect all reagents from light.

1. Allow LDH & ATP substrate & buffer and LDH Stop Solution to come to room temperature on benchtop. Once thawed, prepare LDH Reagent by combining 11 mL Assay Buffer with Substrate and mix. Prepare 1X ATP Reagent by combining Assay Buffer and 2X Substrate and mix, dilute with an equal volume of HBSS or PBS, per manufacturer's instructions.

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2. Allow culture plate to come to room temperature on bench top for ~ 30 minutes prior to assay.
3. Collect 100 µL supernatant from 96-well culture plate, transfer to black walled 96-well plate. Save at room temperature for use in LDH assay.
4. ATP Procedure: Aspirate remaining media from culture plate, and add 200 µL ATP reagent per well, and mix ~ 2 min on an orbital shaker. Incubate at room temperature for 10 minutes, and record luminescence as described in the ATP technical bulletin. See Appendix 3 for the recommended layout.
5. LDH procedure: Use assay plate generated in Step 3 above. Add 100 µL LDH reagent sample wells, shake gently, and incubate at room temperature. Inclusion of 3 Total Lysis wells for normalization of LDH results is considered best practice by Promega. After 10 minutes, add 50 µL Stop Solution per well, including background control wells, shake gently. Record fluorescence (excitation at 560 nm/ emission at 590 nm) as described in the LDH technical bulletin. See Appendix 3 for the recommended layout.

Data Analysis

ATP Depletion Data Analysis

Subtract the background control luminescence from all sample values.

To quantify ATP depletion as a result of treatment, calculate percent of control as the ratio of ATP content of test article treated samples to vehicle control (0.1% DMSO) as described in Equation 1.

BioIVT recommends that all ATP measurements be generated from triplicate wells and be represented by the calculated mean and standard deviation from these biological replicates.

Equation 1:

$$\%\ of\ Control = \frac{ATP\ Content_{Treatment}}{ATP\ Content_{\ 0.1\%\ DMSO}} \times 100$$

LDH Leakage Data Analysis

Subtract blank fluorescence from all sample values.

To quantify LDH Leakage as a result of treatment, calculate percent of control as the ratio of LDH measured for test article treated samples to vehicle control (0.1% DMSO) as described in Equation 2.

BioIVT recommends that all LDH measurements be generated from triplicate wells and be represented by the calculated mean and standard deviation from these biological replicates.

Equation 2:

$$\%\ of\ Control = \frac{LDH\ Measured_{Treatment}}{LDH\ Measured_{\ 0.1\%\ DMSO}} \times 100$$

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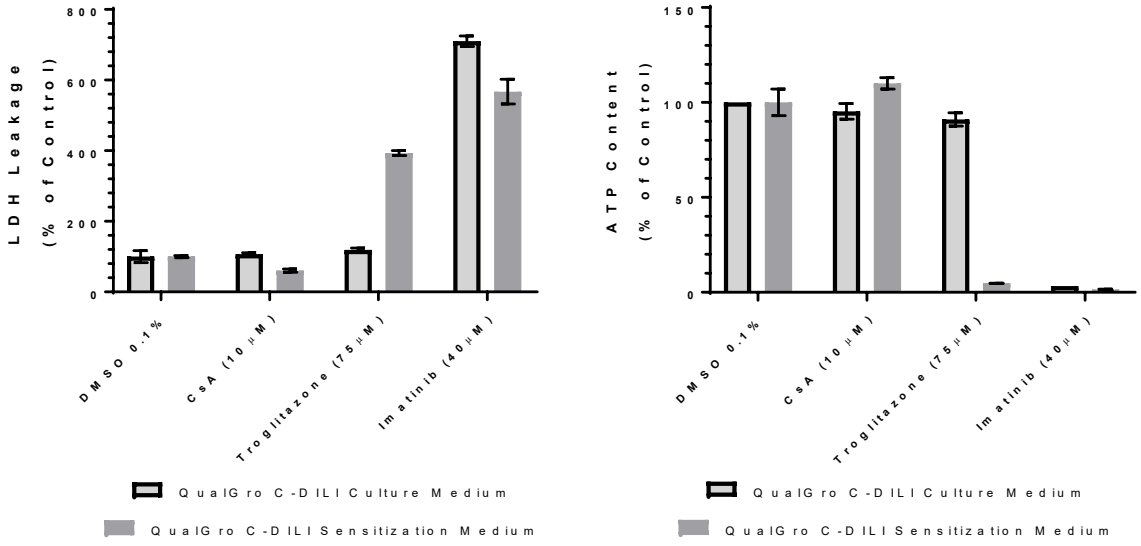
Data Interpretation

The hepatotoxicity mechanism is determined by the LDH leakage and ATP depletion profile across media conditions. Briefly, marked (see key) LDH leakage in only Sensitization Medium (e.g. Troglitazone) suggests a cholestatic hepatotoxicity mechanism while marked LDH leakage in both media conditions (e.g. Imatinib) suggests a general hepatotoxicity mechanism.

DILI Risk Level	
LDH Content Range	**Risk Level**
0-150%	Low
151-199%	Medium
≥200%	High



In the above example data, CsA (a negative control) treatment was not observed to significantly (Dunnett's; p-value > 0.05) increase LDH leakage or decrease ATP content in SCHH under either QUALGRO™ or sensitization culture medium. Troglitazone significantly (Dunnett's; p-value ≤ 0.0001) increased LDH leakage and concomitantly decreased ATP content in cultures under sensitization conditions only. Imatinib treatment significantly (Dunnett's; p-value ≤ 0.0001) increased LDH leakage and reduced ATP under both culture conditions.

It is important to note that released LDH deteriorates over time (half-life ~ 8 hr) and thus early toxicity may present as low LDH leakage for some compounds. In this instance, a low ATP Content relative to control will serve to confirm toxicity.

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Appendix 1: Determining number of viable hepatocytes

Determine the number of viable hepatocytes prior to plating using the following procedures.

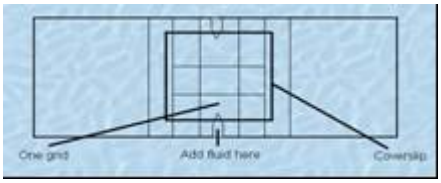
PROCEDURES

1.1. Invert tube of resuspended hepatocytes gently but thoroughly.

1.2. Remove 25 µL of cell suspension in duplicate and place into a 2 ml microcentrifuge tube containing 100 µl 0.05% Trypan blue in DMEM (5-fold dilution, final Trypan blue 0.04%).

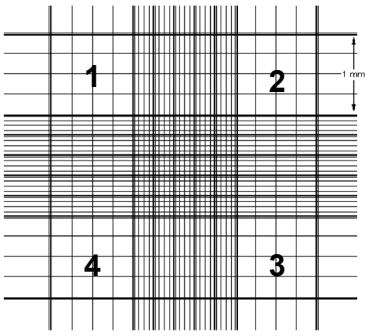
1.3. Place a clean cover slip over the chamber of the hemocytometer.

1.4. Gently rock microfuge tube to mix contents and fill both sides of the chambers with 10 µL of cell suspension (one from each tube) containing Trypan Blue, see figure below.



1.5. View under a microscope using 10X magnification.

(Example of four squares being counted)



1.6. Count the number of viable cells (seen as bright cells) and non-viable cells (stained blue) in all four quadrants of both chambers. Enter into Excel spreadsheet along with volume Seeding Medium cells are suspended in to calculate percentage of viable and non-viable cells and number of cells/mL. Duplicate counts must be within 15% of each other.

1.7. Save an image of the post-thaw cells to the appropriate folder.

1.8. Clean hemocytometer and slide carefully with 70% ethanol and tissue wipe and return to case.

1.8.1. Hemocytometer and cover slips should also be washed regularly with DI H_2O to aid in removal of protein.

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1.9. Calculate % viability:

$$Percentage\ of\ viable\ cells = \frac{Total\ number\ of\ viable\ cells}{Total\ number\ of\ cells\ (viable + nonviable)\ \times 100}$$

1.10. Calculate the number of viable cells/mL:

$$Number\ of\ cells\ per\ mL = \frac{Total\ number\ of\ viable\ cells}{Number\ of\ viable\ cells\ \times\ (dilution\ factor) \times\ 10\text{^}4}$$

1.11. Calculate total number of viable cells:

$$Total\ number\ of\ viable\ cells = \ Number\ of\ viable\ cells\ per\ mL\ \times Total\ liquid\ volume$$

1.12. Calculate total number of cells/vial:

$$Total\ number\ of\ cells\ per\ vial = \frac{Total\ number\ of\ viable\ cells}{Number\ of\ vials\ used}$$

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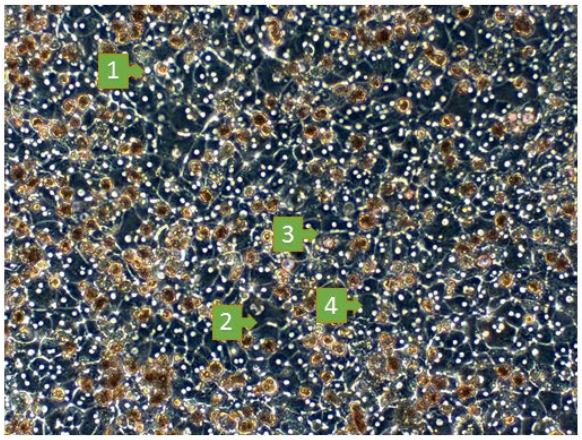
Appendix 2: Comparison of High Versus Low Quality Cultures of Hepatocytes

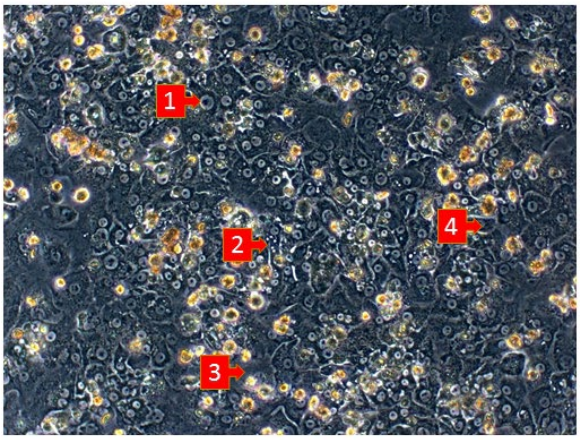
By Day 2 of culture, hepatocytes should begin to polarize and form bile pockets. Cells should be > 90% confluent and 100% confluent is ideal. Below is an example comparing good vs bad cultures of hepatocytes. Poor quality cultures should not be used in the study and should be discarded.

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



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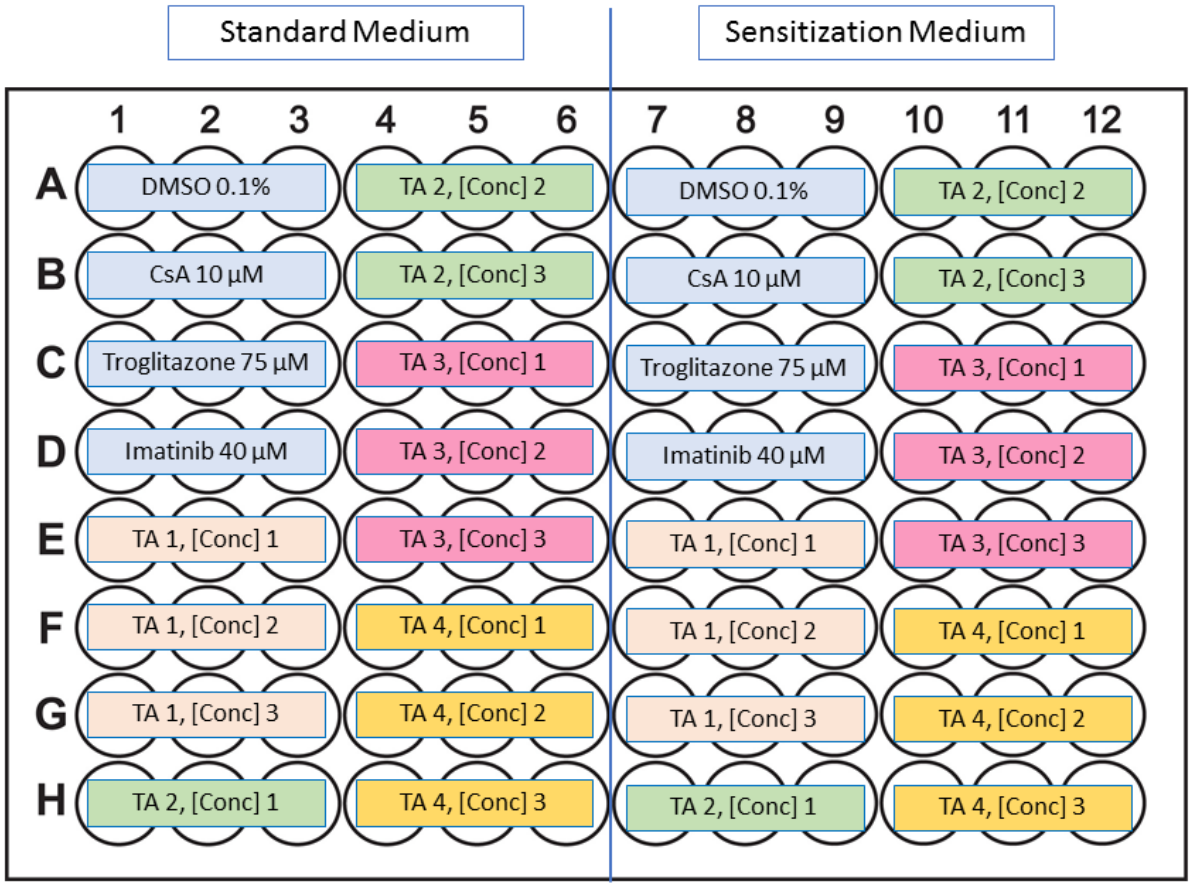
Appendix 3: Experimental Design, Plate Diagram

The diagram below displays dosing for an experimental design using for 4 test articles, at 3 concentrations, with 3 replications. Other experimental designs are feasible with this kit.



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