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Hepatotoxicity Assay

in vitro Screening for Drug-Induced Hepatotoxicity using upcyte® Hepatocytes

2x 48 Assays in 96-well Format

Product # ULC1003-48

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Technical Manual

(v7.2b)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

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in vitro Screening for Drug-Induced Hepatotoxicity using upcyte® Hepatocytes

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I. Description

▪ Drug-Induced Liver Toxicity ▪

The liver, being the main organ responsible for detoxification of xenobiotics, including pharmaceuticals, is itself susceptible to adverse effects of such chemicals or their metabolites. The emergence of liver toxicity is a major reason for the termination of clinical drug trials, as well as the post-market withdrawal of many approved drugs. Consequently, the use of hepatocytes for the early identification of drug candidates that induce acute hepatotoxicity provides a powerful predictive tool that can inform drug development decisions.

▪ Luminescent Reporter Hepatocytes (upcyte®) ▪

This Hepatotoxicity Assay utilizes **upcyte® hepatocytes**, which are human-donor derived hepatocytes established by upcyte® technologies (GmbH)[1-3]. These cells have the attribute of limited proliferation while maintaining their native levels of constitutive and inducible xenobiotic metabolizing enzyme activities. Like primary hepatocytes, confluent cultures of upcyte® hepatocytes express liver-specific proteins, produce urea and store glycogen. Importantly, the induction profiles of cytochrome P450 (CYP) enzyme activities are similar to those of primary hepatocytes. Thus, upcyte® hepatocytes combine the characteristics and advantages of primary hepatocytes with the added practical advantage of having access to the same donor cells for use in iterative, large-scale experiments over extended periods.

These reporter cells are upcyte® hepatocytes derived from donor 10-03, an adult Caucasian female, that have been further modified to constitutively express the luciferase enzyme. The level of luciferase activity expressed in the cells is dependent on the complex coordination of normal cellular processes, including the coupled rates of energy metabolism, transcription, translation, and the turnover of their respective mRNA and protein macromolecules. A drug that perturbs any one of these processes will degrade cell health in a dose- and time-dependent manner, resulting in the reduction of expressed luciferase. Consequently, quantifying relative changes in expressed luminescence intensity between untreated and drug-treated reporter hepatocytes provides an extremely sensitive indicator of emerging cytotoxicity leading to cell death. Importantly, upcyte hepatocytes express, or can induce the expression of, clinically relevant CYPs. Therefore, an administered drug's *de novo* metabolic conversion to a more (or less) toxic form will also be revealed through a corresponding concentration-dependent change in expressed luciferase.

▪ The Assay System ▪

The principle application of this assay is to rapidly screen for test compounds to identify those that induce acute liver cell toxicity.

This kit includes two aliquots of cryopreserved Luminescent Reporter Hepatocytes (upcyte®) prepared using INDIGO's proprietary **CryoMite™** process. As with all cryopreserved mammalian cells, it is imperative that the aliquots of luminescent reporter hepatocytes are stored at temperatures no warmer than -78°C. When properly stored, the cryopreserved cells will yield high cell viability post-thaw (Figure 1) and provide the convenience of immediately dispensing cells into a 96-well assay plate. There is no need for intermediate handling steps such as spin-and-rinse of cells, viability determinations, or cell titer adjustments prior to assay setup. An overnight culture period allows full recovery of the post-thaw hepatocytes and the establishment of a confluent cell monolayer that is ready to receive the user's test compounds.

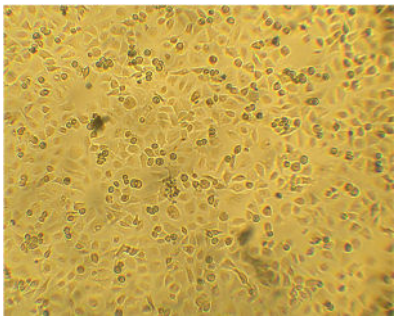


Figure 1. Micrograph of Luminescent Reporter Hepatocytes (upcyte®) Donor 10-03. Cells were thawed and plated following the protocol described in this manual. Representative micrograph taken at 40X, 4h after thaw.

In addition to two aliquots of the reporter hepatocytes, the kit provides two cell culture-ready assay plates, optimized Cell Culture Medium-2 (CCM-2) for use in all steps of the assay procedure (cell thawing, seeding, and preparation of treatment media), luciferase detection reagent, and a reference compound that provides a positive control for hepatotoxicity.

The reagents and materials provided in this assay kit are formatted to allow the user to choose between two alternative assay setups. In one scenario 48 culture wells may be setup at two different times. In the other assay scenario 96 culture wells may be setup at one time.

▪ The Assay Chemistry ▪

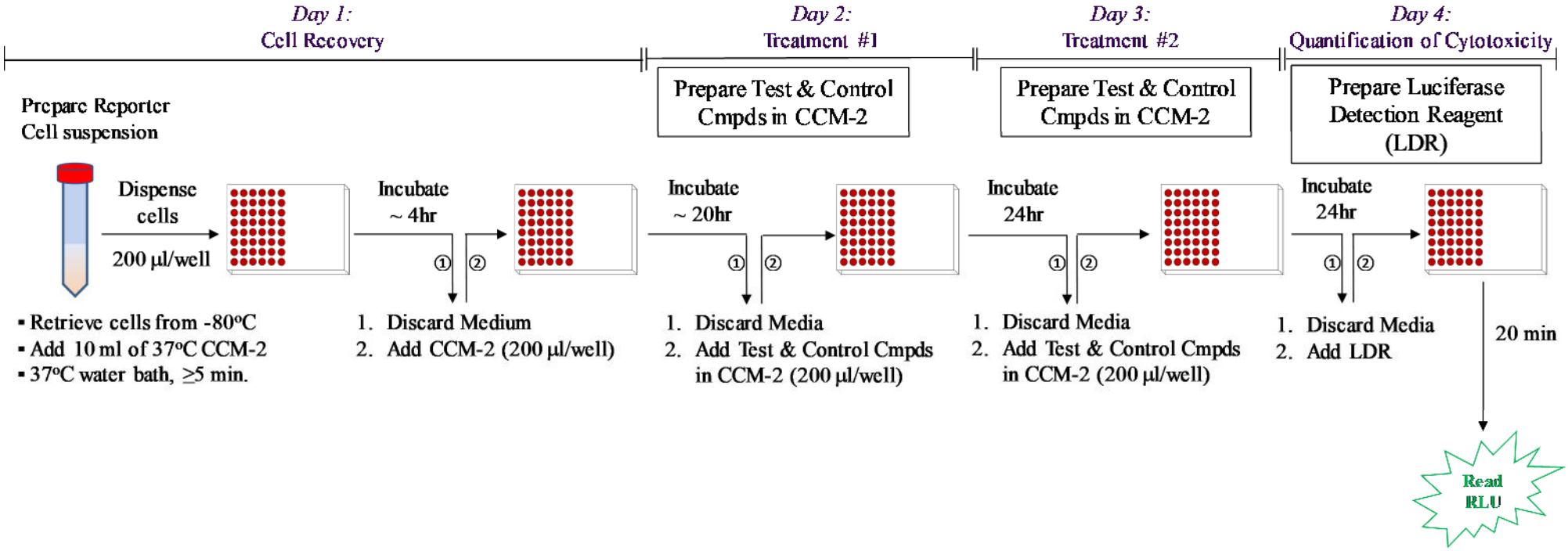
INDIGO's cell-based assay format capitalizes on the extremely low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology.

Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (*Photinus pyralis*). Luciferase catalyzes the mono-oxidation of D-luciferin in a Mg^{+2}-dependent reaction that consumes O_2 and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP_i, CO_2, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer and is reported in terms of Relative Light Units (RLU's).

INDIGO's assay kits feature a luciferase detection reagent specially formulated to provide stable light emission between 5 and 90+ minutes after initiating the luciferase reaction. Incorporating a 20 minutes reaction-rest period ensures that light emission profiles attain maximal stability, thereby allowing assay plates to be processed in batch. By doing so, the signal output from all sample wells, from one plate to the next, may be directly compared within an experimental set.

▪ **Assay Scheme** ▪

Figure 2. Assay workflow for 48hr drug treatment of Luminescent Reporter Hepatocytes (upcyte®).



▪ Preparation of Test Compounds, Reference Compound, Vehicle Control Media ▪

Test and Reference compounds are typically solvated at high-concentration in DMSO and stored frozen as master stocks. To help avert insolubility of compounds at high treatment concentrations, and to limit the amount of DMSO carried over into the culture wells, it is recommended that master stocks are diluted using DMSO to generate a series of 500x-concentrated stocks relative to *each* treatment concentration. In brief, DMSO is used to make serial dilutions of master stocks to generate the desired sub-concentrations that are also 500x-concentrated. Cell Culture Medium-2 (CCM-2) is then used as the final diluent to generate 500-fold dilutions of each DMSO intermediate stock, thereby generating 1x-concentrated treatment media. At *Step 13* of the assay protocol 200 µL of the prepared treatment media are dispensed into respective culture wells containing luminescent reporter hepatocytes. This dilution strategy limits the final concentration of DMSO to 0.2% in each culture well.

If an alternative dilution method is preferred, take care to NOT exceed 0.3% DMSO carried-over into the culture wells, as this concentration will degrade assay performance.

The series of 500x-concentrated stocks prepared in DMSO, and used on Day 2, may be stored at -20°C for later use on Day 3 to prepare the second series of treatment media. Treatment media should always be prepared fresh!

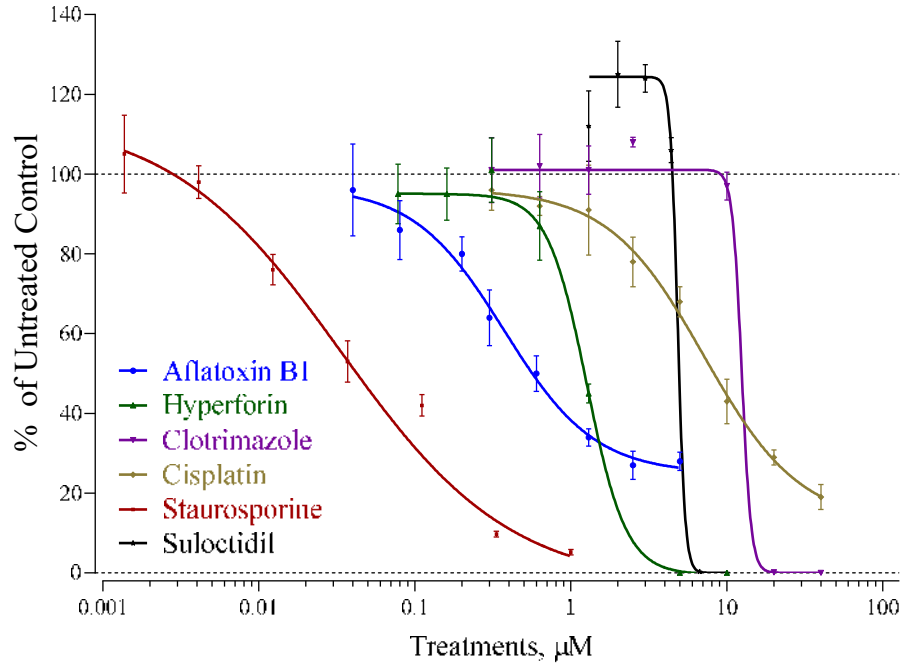
Prepare CCM-2 containing 0.2% DMSO only (*i.e.*, "vehicle only" control media). Drug-induced hepatotoxicity will be determined by comparing RLU in drug treated hepatocytes to RLU in the "vehicle" treated hepatocytes.

▪ Assay Performance ▪

Figure 3 *(next page).* **Dose dependent drug-induced acute cytotoxicity of treated Luminescent Reporter Hepatocytes (upcyte®).** Reporter Hepatocytes were treated for 48hr with known hepatotoxicants. Included are examples of FDA approved drugs, and historical drugs that have subsequently been withdrawn from the market due to their induction of hepatotoxicity. Demonstration of the dose-dependent hepatotoxic potential of Aflatoxin B1, a carcinogen produced by fungi; Staurosporine, a non-selective inhibitor of protein kinases, isolated from Streptomyces; Hyperforin, a potent phytochemical found in the popular nutraceutical St. John's Wort; marketed drugs include Clotrimazole, Cisplatin, Tamoxifen Citrate, Amiodarone, and Irinotecan. Drugs that have subsequently been withdrawn from the market due to hepatotoxicity issues include Suloctidil, Troglitazone, Clozapine, and Perhexiline. Following sequential 24hr + 24hr treatment periods, cells were treated with Luciferase Detection Reagent and luminescence intensity was quantified in terms of relative light units (RLU). Drug-induced toxicity was assessed by normalizing the average RLU values from the individual drug-treatment concentrations to the average RLU from the vehicle treated control hepatocytes. Cell health is assessed by normalizing RLU data to the Vehicle treated Control cells (= 100 %) *vs*. drug concentration. IC_{50} values (expressed in terms of µM) provide measures of relative hepatotoxicant potencies for the various drugs.

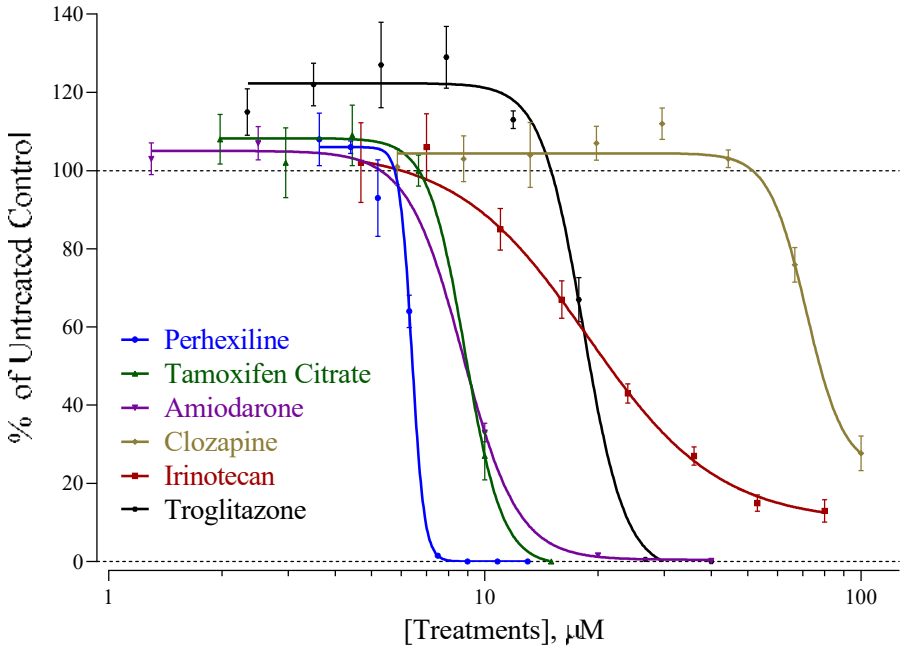
Figure 3

A.



	Aflatoxin B1	Hyperforin	Clotrimazole	Cisplatin	Staurosporine	Suloctidil
IC50, µM	0.3726	1.263	12.45	7.156	0.03337	4.84

B.



	Perhexiline	Tamoxifen Citrate	Amiodarone	Clozapine	Irinotecan (CPT11)	Troglitazone
IC50, µM	6.4	8.865	8.746	71.56	18.73	18.26

II. Kit Components & Storage Conditions

This assay kit contains 2 aliquots of frozen Luminescent Reporter Hepatocytes (upcyte®), thus allowing for two individual cell culture setups comprising 48 wells each. Alternatively, cell suspensions generated from the two individual aliquots may be combined to generate one 96 well assay setup. Two 96-well collagen-coated tissue culture plates are provided to accommodate the user's preferred assay format. If performing a 48 well cell culture setup, *refreeze the extra volumes of all reagents*. The Luminescent Reporter Hepatocytes can NOT be thawed and refrozen.

Assay kits are shipped on dry ice. Upon receipt, individual kit components may be stored at the temperatures indicated on their respective labels. More convenient, however, is to keep kit components together and simply store the entire assay kit in a -80°C freezer.

Please Note: To ensure maximal viability, Luminescent Reporter Hepatocytes must be continuously maintained at no warmer than -78°C until immediately prior to use. Do not allow the tubes of hepatocytes to warm up during kit disassembly; immerse the tubes of cells in dry ice before handling other kit components. Do NOT store cells in liquid nitrogen.

The date of product expiration is printed on the Product Qualification Insert (PQI) enclosed with each kit

Kit Components	***Amount***	***Storage Temp.***
▪ Luminescent Reporter Hepatocytes (upcyte®)	2 x 1.0 mL	**-80°C**
▪ Cell Culture Medium-2 (CCM-2)	1 x 150 mL	-20°C
▪ Tamoxifen Citrate, 500x (7.5 mM in DMSO; positive hepatotoxic reference)	1 x 30 µL	**-80°C**
▪ Detection Substrate	2 x 3.0 mL	**-80°C**
▪ Detection Buffer	2 x 3.0 mL	**-80°C**
▪ 96-well cell culture-ready assay plate (sterile, collagen-coated)	2 plates	-20°C

NOTE 1: This kit contains two 96-well tissue culture plates in which the wells have been collagen-coated and dried; the culture plates should be stored frozen (-20ºC or colder) until use.

III. Materials to be Supplied by the User

The materials listed below must be provided by the user and should be made ready for use prior to commencing the assay procedure.

DAY 1: Cell Recovery

- Dry ice for Protocol *Step 3*
- Cell culture-rated laminar flow hood
- Cell culture incubator (37°C, ≥85% humidity, 5% CO_2)
- 37°C water bath
- 70% alcohol wipes
- 8-channel electronic pipettes capable of repeat-dispensing 200 µl volumes, and sterile tips
- Disposable media basins, sterile
- Waste container for 'media-dump' steps, and clean absorbent paper towels
- *Optional:* clear collagen coated 96-well assay plate, sterile, cell culture treated, for viewing cells.

DAYS 2 & 3: Cell Treatments

- Cell culture-rated laminar flow hood
- Cell culture incubator (37°C, ≥85% humidity, 5% CO_2)
- 37°C water bath
- Dimethyl sulfoxide (DMSO)
- Sterile 96-well PCR plates to prepare 500x stocks of reference and test compounds
- 8-channel manual pipettes (0.5-10 µl) & sterile tips
- 8-channel electronic pipettes capable of repeat-dispensing 200 µl volumes, and sterile tips
- Sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), *or* deep-well plates, *or* appropriate similar vessel for generating 1x-concentrated dilution series of reference and test compounds
- Disposable media basins, sterile
- Waste container for 'media-dump' steps, and clean absorbent paper towels

DAY 4: Cell Harvest

- 8-channel electronic pipettes capable of repeat-dispensing 100 µl volumes, and sterile tips
- Disposable media basins, sterile
- Waste container for 'media-dump' steps, and clean absorbent paper towels
- Plate-reading luminometer

IV. Assay Protocol

Before starting, carefully review the entire assay protocol, as well as the list of "*Materials to be Supplied by the User*" (pg. 9).

DAY 1, Cell Recovery: All steps must be performed using aseptic techniques.

1.) Remove **Cell Culture Medium-2 (CCM-2)** from freezer storage, thaw and equilibrate to **37°C** using a water bath. Mix the solution by inverting the bottle several times.

2.) Remove a **culture-ready plate** from -20°C and place in a 37°C incubator to allow temperature equilibration. If intending to treat 48 wells of Luminescent Reporter Hepatocytes, then only half of one 96-well culture plate will be utilized.

3.) Rapid Thaw of Luminescent Reporter Hepatocytes (upcyte®): *First*, retrieve the bottle of **CCM-2** from the 37°C water bath and sanitize its outside surfaces with a 70% ethanol swab.

Second, if intending to setup **48 treatment wells**: retrieve one tube of **Luminescent Reporter Hepatocytes** from -80°C storage and *immerse the tube in dry ice* to transport it to a laminar flow hood. When ready to proceed, place the tube of cells in a rack and, *without delay*, perform a rapid thaw of the frozen cells by transferring 10 ml from the bottle of pre-warmed **CCM-2** into the tube of frozen cells. Recap the tube and immediately place it in a 37°C water bath for a minimum of 5 minutes. The resulting volume of cell suspension will be 11 ml.

If intending to setup **96 treatment wells**: perform a rapid thaw of each tube of **Luminescent Reporter Hepatocytes**. In *Step 5* the two individually prepared cell suspensions will be combined in a sterile media basin to produce a total volume of 22 ml.

4.) Retrieve the tube(s) of Luminescent Reporter Hepatocytes from the water bath and sanitize the outside surface with a 70% alcohol swab.

5.) *Gently* invert the tube(s) of Luminescent Reporter Hepatocytes several times to disperse cell aggregates and gain a homogenous cell suspension. Caution: do NOT mix the cell suspension by pipetting up and down. Transfer the cell suspension into a sterile media basin. Using a multichannel repeat-dispensing pipette, dispense **200 µl / well** of the cell suspension into the culture plate.

> *NOTE 5.1:* Increased well-to-well variation will occur if care is not taken to prevent cells from settling during the dispensing period. Take care to dispense uniform volumes across the assay plate.
>
> *NOTE 5.2:* Users sometimes wish to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear collagen coated 96-well cell culture assay plate. Continue to process the clear assay plate in identical manner to the white assay plate.

6.) Post-thaw recovery of Luminescent Reporter Hepatocytes (upcyte®): Incubate the assay plate for **~ 4 hr** in a cell culture incubator (37°C, ≥ 85% humidity, 5% CO_2).

> *NOTE:* Ensure a ≥ 85% humidity environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

Near the end of the 4-hour recovery period:

7.) Retrieve the bottle of pre-warmed (37°C) CCM-2 from the water bath and sanitize the outside surface with ethanol wipes.

8.) At the end of the ~ 4 hr cell recovery period, **discard the culture medium** by ejecting it into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets.

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9.) Dispense **200 µl** of CCM-2 into each well and incubate the assay plate for an additional **~ 20 hr** in a cell culture incubator.

NOTE: If well-to-well variation due to 'edge-effects' is a concern this problem *may* be mitigated by dispensing sterile liquid into the *inter-well* spaces of the assay plate. Simply remove 1 tip from the 8-chanel dispenser and dispense 100 µl of sterile water into each of the seven inter-well spaces per column of wells.

DAY 2, First Drug Treatment: All steps must be performed using aseptic

Near the end of the ~20 hr incubation period:

10.) Retrieve the bottle of CCM-2 from refrigerator storage and allow it to equilibrate to room temperature.

11.) Prepare Test and Reference Compounds in CCM-2 to generate '1x-concentrated' treatment media. These treatment media should be prepared immediately prior to adding to the hepatocytes. Manage dilution volumes carefully; this assay kit provides **150 ml** of CCM-2.

NOTE: As discussed in "*Preparation of Test Compounds*" (pg. 6), it is recommended that master stocks are serially diluted in DMSO to generate intermediate stocks at '500x-concentration' relative to *each* final treatment concentration. CCM-2 is then used to make 500-fold dilutions of the DMSO stocks, thereby producing the '1x-concentration' treatment media to be dispensed into respective culture wells (*Step 13*). The 500x-concentrated stocks in DMSO may be stored at -20°C for later use on Day 3 to prepare the second series of treatment media. Do NOT store extra volumes of prepared treatment media; these should be prepared fresh on Day 3.

A Positive Control treatment that induces hepatotoxicity is 15 µM of Tamoxifen Citrate. This reference compound is provided in this kit as a 500x concentrated stock prepared in DMSO. As with the user's test compounds, use CCM-2 to make a 500-fold dilution of the provided Tamoxifen Citrate stock.

NOTE: This recommended strategy for preparing treatment media results in 0.2% DMSO carried over into the culture wells. If an alternative strategy is preferred for making the various treatment media, it is important that the total DMSO carried over into culture wells does **not exceed 0.3%**.

12.) At the end of the ~20hr pre-treatment culture period **discard the culture media,** as described in *Step 8*.

13.) Dispense into respective culture wells **200 µl** of each prepared Reference and Test Compound(s) treatment media. (Refer to the NOTE at *Step 9*.)

14.) Incubate the cells for 24 hr in a cell culture incubator.

DAY 3, Second Drug Treatment: All steps must be performed using aseptic

Near the end of the 24-hour treatment period:

15.) Remove CCM-2 from refrigerator storage and equilibrate to 37°C in a water bath.

16.) Use CCM-2 to prepare fresh **Test Compound** and **Reference Compound treatment media**, as was done in *Step 11* on Day 2.

(continued …)

17.) At the end of the first 24 hr treatment period **discard the culture media,** as described in *Step 8*.

18.) Dispense into respective culture wells **200 µl** of each prepared Reference and Test Compound(s) treatment media. (Refer to the NOTE at *Step 9.*)

19.) Incubate the cells for an additional **24 hr** in a cell culture incubator.

DAY 4, Quantifying Drug-Induced Cytotoxicity: Subsequent manipulations do *not* require special regard for aseptic techniques and may be performed on a bench top.

20.) 30 minutes before intending to quantify hepatotoxicity, remove the Detection Substrate and Detection Buffer from the -80ºC storage and place them in a low-light area so that they may equilibrate to room temperature. If processing one 48-well reaction plate thaw 1 vial each of DS and DB. If processing an entire 96 wells thaw each of the two vials of both DS and DB. Allow solutions to equilibrate to room temperature.

NOTE: Do NOT actively warm Detection Substrate above room temperature; a **room temperature** water bath may be used to expedite thawing.

21.) Set the plate-reader to "luminescence" mode. Set the instrument to perform a single 5 second " plate shake" prior to reading the assay well. Read time may be set to 0.5 second (500 mSec) per well, or less.

22.) Immediately before proceeding to Step 23, transfer the entire volume(s) of Detection Buffer into the vial(s) of Detection Substrate and gently invert to ensure homogenous mixtures of **Luciferase Detection Reagent (LDR)**. The final reatgent volume will be either 6 ml (for 48 wells) or, after combining the separate LDR preparations, 12 ml (for processing 96 wells).

23.) At the end of the second 24hr treatment period **discard the culture media,** as described in *Step 8*.

24.) Add **100 µl** of **LDR** to each well of the assay plate. Allow the assay plate to rest at room temperature for at least 20 minutes following the addition of LDR. Do not shake the assay plate during this period.

25.) Quantify luminescence.

V. Limited Use Disclosures

upcyte® Hepatocytes are used by INDIGO Biosciences through commercial license agreement with upcyte technologies, GmbH (Hamburg, Germany).

Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic, diagnostic, or any form of contact application use in humans or animals.

"CryoMite" is a Trademark ™ of INDIGO Biosciences, Inc. (State College, PA, USA)

Product prices, availability, specifications and claims are subject to change without prior notice.

VI. References

[1] Burkard A. *et. al.* (2012) Generation of proliferating human hepatocytes using upcyte® technology: characterization and applications in induction and cytotoxicity assays. Xenobiotica **42** (10): 939-956.

[2] Ramachandran SD. et. al. (2015) Applicability of second-generation upcyte® human hepatocytes for use in CYP inhibition and induction studies. Pharma Res Per **3** (5): e00161.

[3] Tolosa L. *et. al.* (2016) Human Upcyte Hepatocytes: Characterization of the Hepatic Phenotype and Evaluation for Acute and Long-Term Hepatotoxicity Routine Testing. Toxicological Sciences **152** (1): 214-229.