Lonza

Development of a Long-term Primary Human Hepatocyte 3D Spheroid Model

For Use in Drug-induced Liver Injury (DILI) Applications Using ViaLight® Plus Cytotoxicity BioAssay Kit

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Abstract

3D primary human hepatocyte cell culture models provide a more physiologically-relevant *in vitro* system for evaluating drug-induced liver injury (DILI) compared to 2D models. Here, we discuss the development of a long-term primary human hepatocyte 3D spheroid model that is suitable for either acute or chronic toxicity assays in a 96-well, high-throughput format. We successfully optimized a protocol for a 28-day culture during which spheroids were formed on day 7. We tested the resulting spheroids in a chronic drug toxicity assay by repeatedly exposing them to acetaminophen for 14 days after spheroid formation, measuring viability at multiple time points using Lonza's ViaLight® Plus Cytotoxicity BioAssay Kit and validating the results. We found that EC50 values generated by the ViaLight® Plus Cytotoxicity BioAssay Kit were comparable to published literature values, demonstrating that the spheroid model performed as expected across all time points. The resulting protocol, when used with the ViaLight® Plus Cytotoxicity BioAssay Kit, provides a highly sensitive, user-friendly DILI model that can be integrated into automated, high-throughput screening workflows.

Introduction

The recently enacted FDA Modernization Act 2.0 reinforced the need to develop and refine *in vitro* models and cell-based assays for hepatotoxicity as an alternative to animal testing during pre-clinical phases of drug discovery and development. A wide variety of models for evaluating drug-induced liver injury (DILI) exist[1], but they require development and refinement to match the viability of animal models. Unfortunately, the most physiologically relevant models are also the most complex.[1-3] With optimization being both time-consuming and expensive, one alternative, the 3D spheroid primary human hepatocyte (PHH) cell model, is emerging as a viable approach for evaluating toxicity. This model:

- Provides a much closer approximation to *in vivo* conditions when compared to 2D models.[2,4]
- Is readily adaptable to high-throughput workflows.
- Provides critical assessment of the efficacy of cytotoxic or cytoprotective compounds, when used in conjunction with ATP-based endpoint assays of cell viability.

In this white paper, we present a long-term, 3D spheroid PHH culture that is user-friendly, scalable to high-throughput screening applications, and suitable for both acute and chronic hepatotoxicity assessment.[5] When used in conjunction with the Lonza ViaLight® Plus Cytotoxicity BioAssay Kit – an ATP-based luminescence assay – we obtained EC50 values that matched expected values from literature. Overall, we developed a robust, physiologically-relevant method for determining the efficacy of cytotoxic or cytoprotective compounds that is easily scalable to automated, high-throughput workflows.

Key Takeaways

- Our 3D spheroid PHH culture maintained healthy morphology and physiology through 28 days in culture.
- The spheroids behaved as expected in a cell-based, chronic hepatotoxicity assay using acetaminophen.
- The Lonza ViaLight® Plus Cytotoxicity BioAssay Kit provides a validated, highly sensitive, user-friendly end-point assessment of DILI.

Materials and Methods

Materials:

Table 1 summarizes the materials used in the optimized, long-term, 3D spheroid PHH culture, as well as for the chronic hepatotoxicity assay with endpoint analysis.

Vendor	Catalog No.	Description
Lonza	HUCPI	Cryopreserved Primary Human Hepatocytes, Plateable, DDI Qualified. (Verified for Spheroids)*, ≥ 5 million cells
Lonza	MCHT50	Human Hepatocyte Thawing Media, 50 mL
Lonza	CC-3199	HBM Basal Medium, 500 mL
Lonza	CC-4182	HCM SingleQuots® Supplements
Lonza	CC-3198	HCM Hepatocyte Culture Medium BulletKit®
Lonza	BEBP17-737E	1 M HEPES Buffer, 100 mL
Lonza	LT07-321	Lonza ViaLight® Plus Cytotoxicity BioAssay Kit, 10,000 tests
Lonza	LT07-121	Lonza ViaLight® Plus Cytotoxicity BioAssay Kit, 1,000 tests
Lonza	LT07-221	Lonza ViaLight® Plus Cytotoxicity BioAssay Kit, 500 tests
Lonza	LT27-008	ATP Standard, 5 mL
Corning®	CLS7007	96-Well Ultra-Low Attachment Plates
Corning®	3912	96-Well White Luminescence Plates
Any		FBS
Any		Trypan Blue 0.4%
Any		Acetaminophen
Any		Luminometer

**Verified for Spheroids: Lonza routinely screens plateable hepatocyte lots for spheroid formation potential. To learn which lots are characterized for spheroid formation, contact Technical Support at scientific.support@lonza.com or scientific.support.eu@lonza.com.*

Table 1. Materials used by Lonza to create the 3D spheroid PHH culture and test hepatotoxic response to acetaminophen.

3D Spheroid PHH Culture

Detailed instructions for establishing the optimized, long-term, 3D spheroid cultures of Lonza PHH can be found in the protocol published on the Lonza website.[5] Briefly summarized, we prepared a "Spheroid Formation Medium" prior to the experiment by combining the complete Lonza Hepatocyte Culture Medium (HCM) – prepared according to manufacturer instructions – with FBS and HEPES, making the final concentrations of each 20% and 25 mM, respectively. To thaw the Lonza PHHs (part no. HUCPI; n =

3 donors), we used warm Hepatocyte Thawing Medium, in which we carefully suspended cells, centrifuged, and then aspirated.

Next, we re-suspended the cells in 3 mL of pre-warmed Spheroid Formation Medium, counted them using Trypan Blue and a hemocytometer at a 1:5 dilution, then adjusted them to a final concentration of 1.5 x 10^4 cells/mL in Spheroid Formation Medium. We plated the cells in an ultra-low attachment 96-well plate at a density of 1,500 cells/well (100 µL of cell suspension). To control evaporation during the spheroid formation step, we left an outer ring of wells around the cells to fill with Spheroid Formation Medium.

Once the plates were prepared, we incubated them in a humidified incubator at 37°C, 5% CO_2 for 5 days, during which time they were left undisturbed (i.e., no medium changes, etc.). On days 5 and 6, we aspirated 50% of the medium from each well – taking care to not pipette out spheroids – then replaced it with fresh, pre-warmed, serum-free Lonza HCM, prepared as per the manufacturer's instructions.

Hepatoxicity Model with Acetaminophen as Toxicant

On day 7 of PHH culture (at which point spheroids had formed), we prepared 2X Drug Dosing Medium (double-strength) by dissolving acetaminophen in HCM medium at a 50 mM concentration (for a desired highest dose of 25 mM) and then titrated to produce eight different concentrations ranging from 0 – 50 mM. While any drug compound can be used for this protocol, we chose acetaminophen as it is a well-characterized hepatotoxic compound suitable for DILI models. We carefully aspirated 50% of the medium from each well and then replaced it with 2X Drug Dosing Medium, resulting in a final concentration of 1X. Next, we returned cells to the incubator and cultured them for an additional 21 days (totaling 28 days in culture), with 50% medium changes performed every 2 – 3 days using 1X Drug Dosing Medium (acetaminophen dissolved in HCM medium at a concentration of 25 mM and titrated to eight different concentrations ranging from 0 – 25 mM).

End-Point Analyses

We visually assessed the morphological health of 3D spheroid PHHs using phase-contrast microscopy. On days 8, 10, 14, 21, and 28 in culture, we harvested spheroids for total cellular ATP, albumin, and CYP3A4 activity, helping us to determine the overall health and functionality of the 3D spheroid culture (in the drug-free control condition).

- **ATP:** Measured using an industry-leading ATP assay
- **Albumin:** Measured via ELISA assays of media taken from harvested wells
- **CYP3A4 Activity*:** Measured using the Promega® P450-Glo™ Assay following manufacturer's instructions via a luminometer

* Basal vs. induced after 72 hours treatment with 10 µM rifampicin in DMSO.

We measured all endpoints on n = 2 – 6 individual spheroids per condition at each timepoint.

In a follow-up experiment, using the optimized and validated culture and drug dosing protocol, we assessed 3D spheroid PHHs for total cellular ATP on days 10, 14 and 21 in culture (days 3, 7 and 14 post-drug dosing). Here, we compared the Lonza ViaLight® Plus Cytotoxicity BioAssay Kit against the industry-leading ATP assay used in the first set of experiments. When developing the protocol, we found that the original ViaLight® Plus Cytotoxicity BioAssay Kit protocol generated an insufficient signal-to-noise ratio due to the much smaller overall number of cells in hepatocyte spheroids (thousands) compared to other cell culture types (e.g., tumor spheroids with > 10,000 cells). Consequently, we developed an optimized protocol **published on the Lonza website**.[6] To summarize this protocol, we began by diluting an ATP standard to a series of eight concentrations ranging between 0 and 10 µM. Next, we removed 50 µL of medium from each well in the 96-well spheroid plate – carefully as to not pipette out any spheroids. We added 50 µL of each ATP standard to a white assay plate, followed by 25 µL ViaLight® Plus Cytotoxicity BioAssay Kit Cell Lysis Reagent in each ATP standard well in the white assay plate and in each spheroid well in the original sample plate. We incubated both plates for 30 minutes at room temperature, after which we added 75 µL ViaLight® Plus Cytotoxicity BioAssay Kit AMR Plus reagent to each sample plate well and assay plate standard well. Finally, we transferred all the contents (approximately 150 µL) from each sample well to a new well on the white assay plate, incubated them for two minutes, then read luminescence on a luminometer. Using these methods, we measured n = 4 – 6 individual spheroids per condition per time point.

EC50 Calculation

We normalized data to the respective vehicle control on each day, and generated dose response curves using log (drug concentration) vs. normalized response with a 3PL fit. This model forces the curve to run from 100% ATP (relative to control) down to 0%, with the EC50 value being the concentration that provokes a response equal to 50%.

Results

3D Spheroid PHHs Last at Least 28 Days in Culture

We compared the optimized 3D spheroid PHH culture conditions described above against a commonly used culturing medium (Williams E) and a slightly modified version of Lonza HCM medium (supplemented with 10% FBS, no HEPES, and gentamycin/amphotericin replaced with gentamycin only). Compared to the other two media types (data not shown), Spheroid Formation Medium promoted the best morphological and physiological endpoints. In this medium, spheroids from all three donors formed by day 7 and maintained stable morphology through day 28 (Figure 1). Additionally, cell functionality – as determined

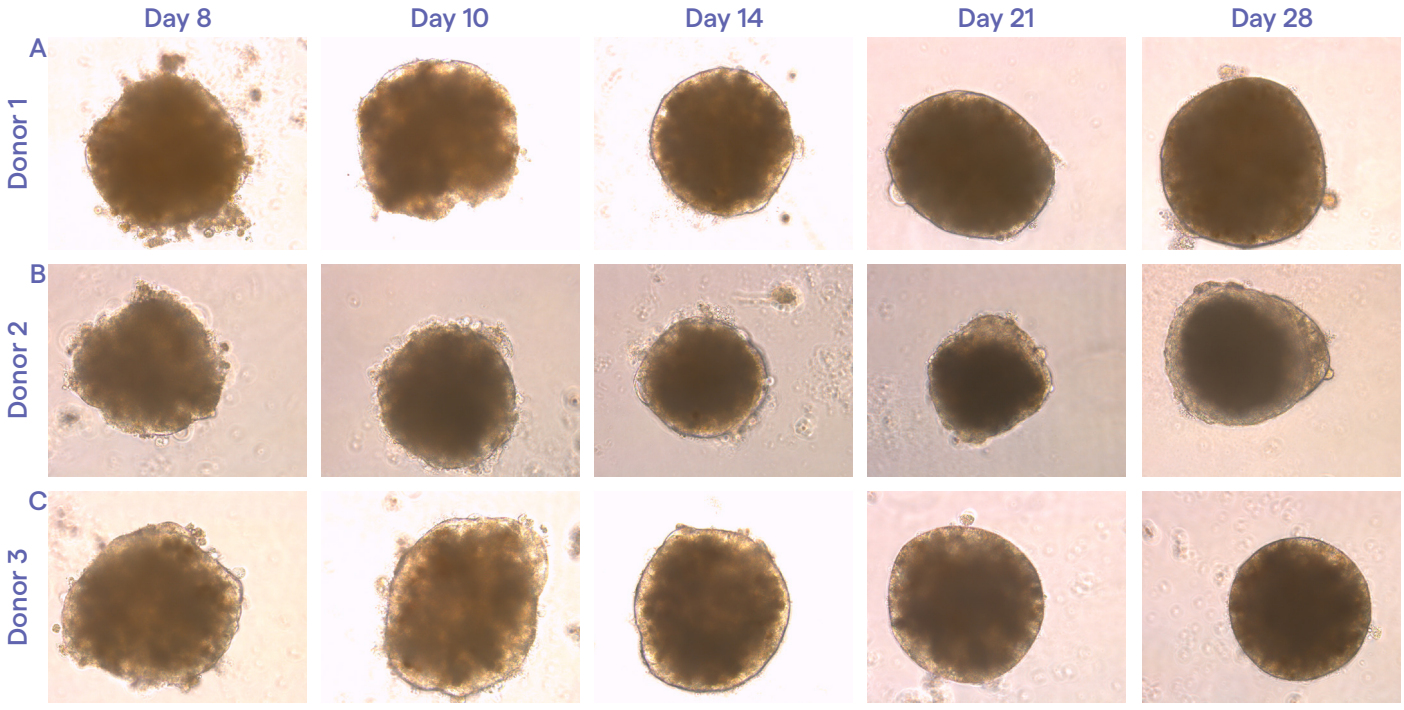

Figure 1.
Phase contrast images of spheroids from Donor 1 (A), Donor 2 (B), and Donor 3 (C) at five time points across a long-term culture. Spheroids were formed in all three donors as of day 7 (not shown).

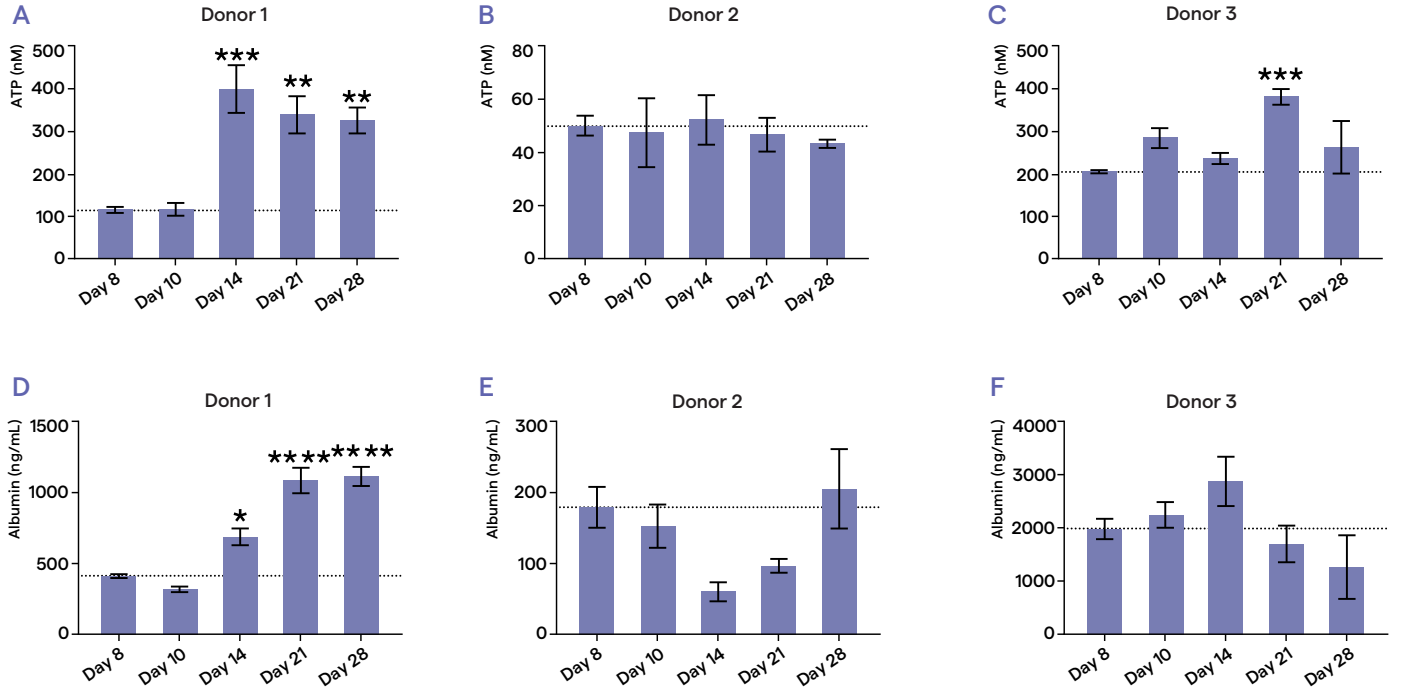

Figure 2.
Cell functionality of 3D spheroid PHHs from three donors at five time points across a long-term culture as measured by cellular ATP (A – C) and albumin (D – F). The dotted line indicates ATP or albumin levels at day 8 of culture, considered to be the first day of spheroid culture, and indicates if ATP or albumin levels are maintained throughout the length of the spheroid culture.

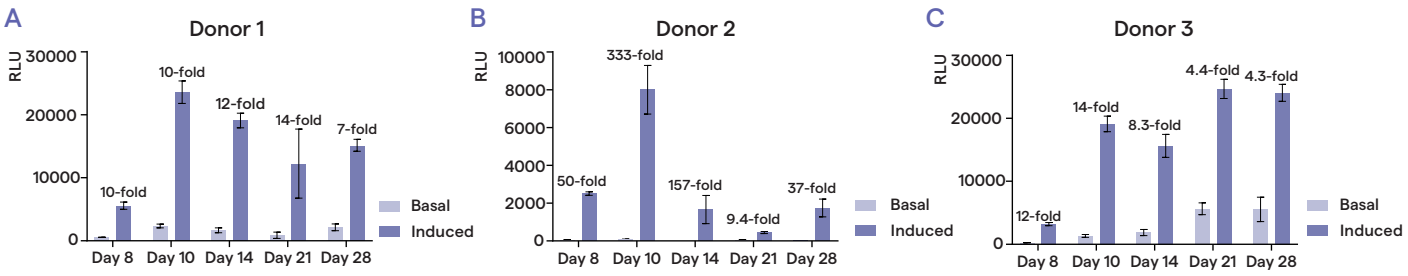

Figure 3.
Basal (light purple bars) and rifampicin-induced (dark purple bars) CYP3A4 levels for hepatocyte Donor 1 (A), Donor 2 (B), and Donor 3 (C) throughout a long-term 3D spheroid PHH culture. Fold-changes given above dark purple (induced) bars are relative to the basal (light purple) condition. RLU = Relative Light Units.

through cellular ATP and albumin production – remained mostly stable throughout the entire 21-day period (Figure 2) except for albumin production by Donor 2, which fell during the first week after spheroid formation, but then recovered (Figure 2E).

CYP3A4 induction remained stable throughout the entire 28-day culture for all three donors (Figure 3). Again, donor 2 maintained a much lower basal activity than the other two donors – but showed the largest response to rifampicin induction (Figure 3B).

Hepatotoxic Response and Validation of ViaLight® Plus Cytotoxicity BioAssay Kit in 3D Spheroid PHHs

All three donors showed a chronic hepatotoxic response to prolonged exposure to acetaminophen, with EC50 values dropping steeply by day 7 of exposure (Figure 4). When

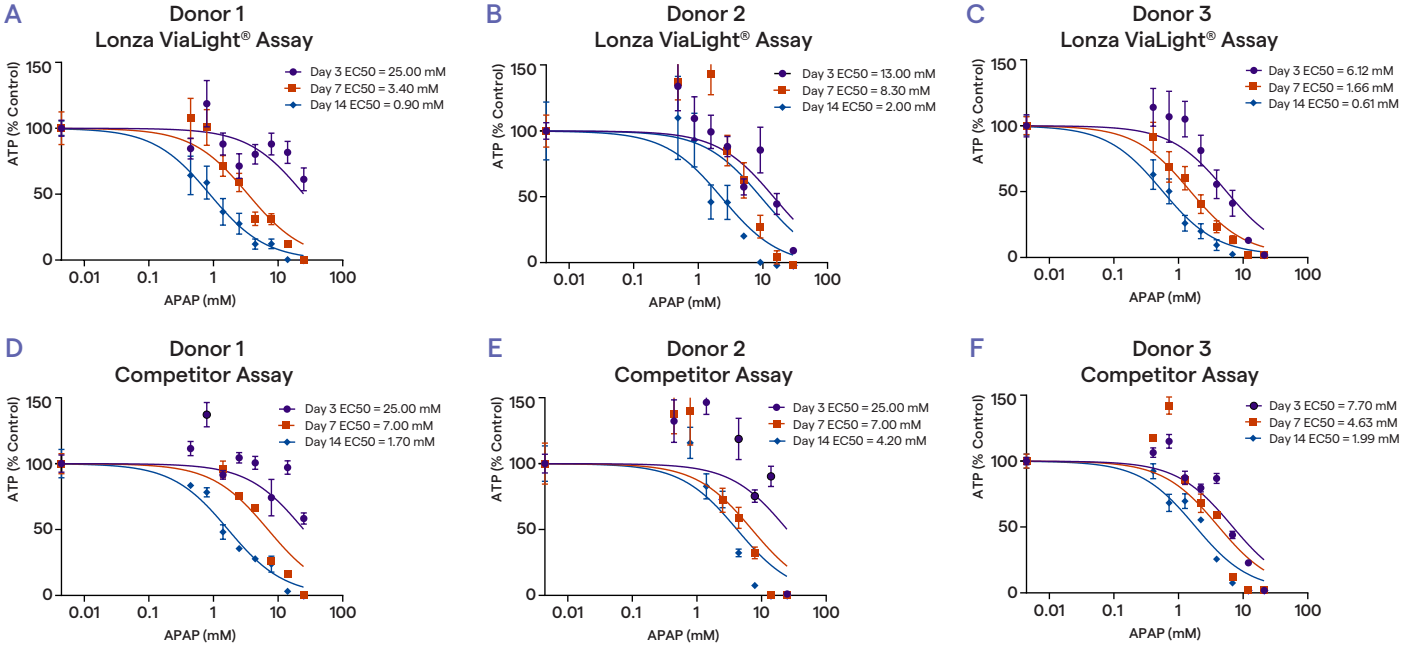

Figure 4.
Validating Lonza's ViaLight® Plus Cytotoxicity BioAssay Kit (A – C) using a modified protocol for 3D spheroid PHHs against the industry-leading cellular ATP assay (D – F) across three donors.

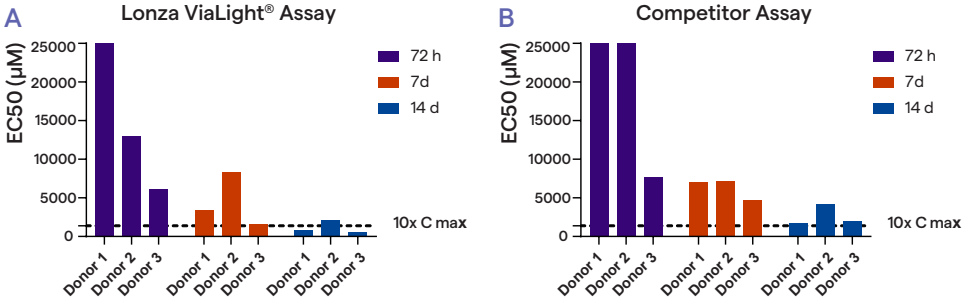

Figure 5.
Comparison of EC50 values for acetaminophen after 3, 7 and 14 days of chronic exposure in a 3D spheroid PHH model as determined by Lonza's ViaLight® Plus Cytotoxicity BioAssay Kit (A) and the industry-leading cellular ATP assay (B). Dashed lines indicate 10x therapeutic Cmax concentrations for acetaminophen (human Cmax values: 139 µM).[2]

compared to the leading cellular ATP assay, Lonza's ViaLight® Plus Cytotoxicity BioAssay Kit used with a modified protocol consistently gave lower EC50 values (Figure 4). As a result, ViaLight® Plus Cytotoxicity BioAssay Kit better reflected the expected 10x Cmax values for acetaminophen[2] at both day 7 and day 14 across all three donors when compared against the leading assay (Figure 5).

Discussion

Our results provide a long-term culture protocol for 3D spheroids derived from Lonza PHHs that maintain desired morphology and physiology through 28 days in a high-throughput format. The model functions as expected across all time points when used as a DILI model for chronic hepatotoxicity, with EC50 values for all three donors falling into well-characterized ranges for acetaminophen.[2] While we observed donor-to-donor variation in physiology and morphology (e.g., Donor 2 vs Donors 1 and 3; Figures 1 – 2), this variation did not impact the overall performance of the 3D spheroid PHHs in the chronic hepatotoxicity assay (Figures 4 – 5).

More importantly, donor-to-donor differences in spheroid physiology and viability – both in general culture and under chronic hepatotoxic conditions – can be related back to hepatocyte donor characteristics. Variation between donors could therefore be explained by phenotypic differences between donors, and not random experimental variation. For example, low baseline ATP and decreased albumin production in spheroids formed by Donor 2 hepatocytes (Figure 2B and 2E) could be partially explained by the overall health of the donor with a BMI of 30.2 (Table 2). The other two donors had much lower BMIs (18.4 and 24.7 for Donors 1 and 3, respectively), which could explain their higher ATP and albumin production.[7,8] Nevertheless, after 28 days in culture, Donor 2 exhibited an overall healthy physiology. In addition, Donor 3 exhibited much higher acetaminophen toxicity at earlier time points (Figure 4C and 4F) than the other two donors, which could be directly correlated with high levels of CYP2E1 enzyme activity found in this donor during initial donor characterization in the production process (Table 2).

Interestingly, CYP2E1 activity correlates with increased acetaminophen-mediated toxicity due to the formation of toxic liver metabolites.[2] High CYP3A4 induction similarly correlates with acetaminophen-mediated toxicity, though to a lesser extent.[9] The combined donor characteristics shown in Table 2 could help explain the variation between donors in CYP34A induction, viability, DILI, and other physiological characteristics observed in our 3D spheroid PHH model.

Our results also demonstrated that the optimized and validated version of the ViaLight® Plus Cytotoxicity BioAssay Kit outperformed the industry-leading cellular ATP assay, proving to be both highly-sensitive and better at matching published physiological responses for 3D PHH spheroids in an acetaminophen-based DILI model[2] over the entire time course of the long-term culture (Figure 5). The ViaLight® Plus Cytotoxicity BioAssay Kit is easily adaptable to high-throughput applications and is an excellent choice for use as an end point assay in DILI applications with 3D spheroid PHHs.[6]

Using reported protocols[5,6], we developed a long-term, physiologically-relevant 3D spheroid PHH model for both acute and chronic hepatotoxicity. Additionally, we demonstrated highly-sensitive, cost-effective, and user-friendly endpoint analyses using an optimized protocol for the ViaLight® Plus Cytotoxicity BioAssay Kit.

Together, these solutions provide researchers with plug-and-play tools to address high-throughput screening phases of drug discovery research with a biologically relevant model. They also significantly reduce the need for *in vivo* animal models during drug development, while at the same time enabling researchers to meet regulations dictated by the FDA Modernization Act 2.0.

Lonza offers primary human hepatocytes in a variety of formats, including plateable and suspension. Plateable human hepatocytes are characterized for induction potential, transporter activity and enzyme activity, and are screened for long-term culture, spheroid formation, and 96-well compatibility.

Want to find products that best meet your cell-based and drug-discovery assay needs?

Contact Lonza Technical Support (scientific.support@lonza.com or scientific.support.eu@lonza.com).

Lot #	Donor #	Gender	Race	Age	BMI	Suspension Metabolism CYP2E1	2D CYP3A4 Induction (Fold Change)	3D CYP3A4 Induction (Fold Change), Day 10	Acetaminophen EC50 (mM), Day 10
HUM200101	1	Male	Asian	63	18.4	2.8	13	10	25
HUM222051	2	Male	Caucasian	30	30.2	5.4	17	333	13.03
HUM222621	3	Female	Caucasian	49	24.7	6.7	N/A*	14	6.117

Table 2.
Select characteristics for the three primary human hepatocyte donors utilized in this study. Fold Change = fold change from basal enzyme levels. Day 10 refers to the 10th day of hepatocyte culture, or three days after spheroid formation and the first dose of acetaminophen. N/A* = this donor failed CYP3A4 induction in a 2D format during quality control, but performed normally in a more physiologically-relevant 3D format.

References

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Ordering Information

Catalog No.	Description	Size
HUCPG	Cryopreserved Primary Human Hepatocytes, Plateable (Verified for Spheroids)*	≥ 5 million cells
HUCPI	Cryopreserved Primary Human Hepatocytes, Plateable, DDI Qualified (Verified for Spheroids)*	≥ 5 million cells
MCHT50	Human Hepatocyte Thawing Media	50 mL
CC-3199	HBM Basal Medium	500 mL
CC-4182	HCM SingleQuots® Supplements	1 kit
CC-3198	HCM Hepatocyte Culture Medium BulletKit®	1 kit
BEBP17-737E	1M HEPES Buffer	100 mL
LT07-321	Lonza ViaLight® Plus Cytotoxicity BioAssay Kit	10,000 test kit
LT07-121		1,000 test kit
LT07-221		500 test kit
LT27-008	Lonza ATP Standard	5 mL

***Ask for lots Verified for Spheroids:** Lonza routinely screens plateable hepatocyte lots for spheroid formation potential. To learn which lots are characterized for spheroid formation, contact Technical Support at scientific.support@lonza.com or scientific.support.eu@lonza.com.

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