

Primary mouse hepatocyte 3D spheroids

**High-throughput, physiological
relevance for drug safety and
toxicology testing**

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Introduction

While guidance provided by the FDA Modernization Act 2.0 indicates an eventual shift from animal studies towards physiologically-relevant *in vitro* human cell culture models, rodent systems will continue to play an important role in safety and toxicology testing for years to come. In the meantime, to align with the goal of minimizing the use and impact of testing on animals, the use of more physiologically-relevant 3D cell culture formats, such as mouse hepatocyte spheroids, can offer better predictivity of *in vivo* results in live animals.¹ Rodent cell culture models require the sacrifice of far fewer animals than *in vivo* animal studies, and early detection of toxicity *in vitro* can ultimately reduce animal use across the entire testing phase, making physiologically relevant cell culture models a priority.

Previous studies have shown that culturing mouse hepatocytes in 3D supports strong physiological relevance and allows cultures to be sustained long enough to assess both acute and chronic toxicity.^{2,3} In this study, we present a protocol for long-term culturing of two strains of Lonza mouse hepatocytes (CD-1 and C57BL/6) in a 3D spheroid format. This workflow is user-friendly, high-throughput, and is suitable for both acute and chronic drug-induced liver injury (DILI) assessment. We further demonstrate the utility of this model through a DILI assessment of acetaminophen, an exemplar hepatotoxin.

Methods

Detailed instructions for establishing the optimized 3D spheroid mouse hepatocyte culture model can be found in the protocol [published on the Lonza website](#).⁴ Materials used in this study can be found in Table 1.

Briefly, we first prepared a Spheroid Formation Medium that consisted of Lonza Hepatocyte Culture Medium (HCM™ Medium) with 20% FBS and 25 mM HEPES buffer to

encourage spheroid formation. Next, two strains of Lonza mouse hepatocytes — CD-1 (part no. M CCP01) or C57BL/6 (part no. M BCP01) — were thawed in Lonza Rodent and Monkey Cryopreserved Hepatocyte Thawing Medium (part no. M CRT50). After thawing, tubes were centrifuged and the supernatant aspirated, after which cells were resuspended in Spheroid Formation Medium.

Next, hepatocytes were counted using 0.4% trypan blue and a hemocytometer, then adjusted to a final concentration of 1×10^6 cells/mL. An aliquot was diluted to 15,000 cells/mL using Spheroid Formation Medium and dispensed at 100 μ L per well in an ultra-low attachment 96-well plate to a final cell seeding density of 1,500 cells/well. Plates were incubated at 37 °C and 5% CO₂ in a humidified incubator and left undisturbed for 5 days. On days 5 – 7, 50% medium changes were performed using fresh, pre-warmed HCM™ Medium. Spheroids formed by day 7 and were maintained in culture for up to 21 days, with 50% medium changes every 2 – 3 days.

DILI toxicity model with acetaminophen

On day 7 of culture (by which point spheroids had formed), we prepared a 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 in the assay), then titrated to produce eight different concentrations ranging from 0 – 50 mM. We carefully aspirated 50% of the medium from each well and replaced it with 2X Drug Dosing Medium, resulting in a final concentration of 1X the intended dosage in each well. Cells were returned to the incubator and cultured for an additional 14 days (totaling 21 days in culture). We performed 50% medium changes every 2 – 3 days using a 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) maintaining the proper dose per-well throughout the culture period.

End point analyses for protocol optimization included visual assessment of spheroid morphology using phase contrast microscopy together with measurements of total

Vendor	Catalog no.	Description	Contents
Lonza	MCCP01	Cryopreserved CD-1 Mouse Hepatocytes, Plateable	≥ 5 million cells
	MBCP01	Cryopreserved C57BL/6 Mouse Hepatocytes, Plateable	≥ 5 million cells
	MCRT50	Rodent and Monkey Cryopreserved Hepatocyte Thawing Medium	50 mL
	CC-3199	HBM™ Basal Medium	500 mL
	CC-4182	HCM™ SingleQuotes® Supplements	1 kit
	CC-3198	HCM™ Hepatocyte Culture Medium BulletKit®	1 kit containing HBM™ + SingleQuotes®
	BEBP17-737E	1M HEPES Buffer	100 mL
	LT07-321	Lonza ViaLight® Plus BioAssay Kit	10,000 test kit
	LT07-121	Lonza ViaLight® Plus BioAssay Kit	1,000 test kit
	LT07-221	Lonza ViaLight® Plus BioAssay Kit	500 test kit
Corning®	CLS7007	96-Well Ultra-Low Attachment Plates	
Corning®	3912	96-Well White Luminescence Plates	
Any		FBS	
		Trypan Blue 0.4%	
		Acetaminophen	
		Luminometer	

Table 1. Materials used to create the 3D spheroid mouse hepatocyte cultures and test hepatotoxic responses to acetaminophen.

cellular ATP and Cyp3a11 activity (72-hour induction with 10 μ M rifampicin). Cyp3a11 activity was measured via an industry-standard luminescence-based assay. For the DILI toxicity assay using acetaminophen, we quantified cellular ATP (using the Lonza Vialight[®] Bioassay Kit) as a proxy for cell viability. All experimental results are from 3 experiments using 3 different lots of mouse hepatocytes per strain. For all spheroid cultures, ATP and Cyp3a11 activity were measured at days 10, 14 and 21 of culture (representing 3, 7, and 14 days post-drug exposure).

Results:

Optimized mouse hepatocyte spheroid culture

Primary mouse hepatocytes from both strains (CD-1 and C57BL/6) formed spheroids with well-defined perimeters within 7 days and maintained appropriate morphology for an additional 2 weeks in culture (total of 21 days; Figure 1). Cyp3a11 induction by rifampicin was observed across the entire 14 day spheroid culture period in both

DILI Toxicity Model with Acetaminophen

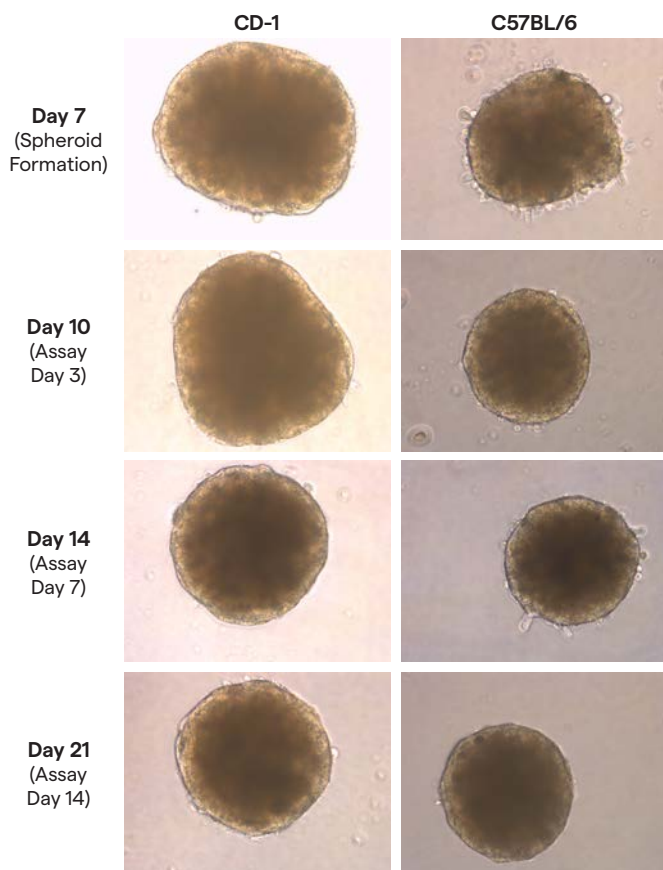


Figure 1. Representative phase-contrast photomicrographs of CD-1 and C57BL/6 mouse hepatocyte spheroids. Spheroids had formed by day 7 and were cultured for a further 14 days (21 in total). 'Assay Day' refers to days 3, 7, and 14 post-drug exposure, when endpoint measurements for the acetaminophen DILI assay were taken. Images are at 20X magnification.

mouse strains (Figure 2); however, we observed that basal Cyp3a11 activity was far higher in strain CD1, resulting in an overall lower induction potential (difference between basal and induced states; Figure 2A) compared to the C57BL/6 strain (Figure 2B) but much higher activity in general. Overall, both mouse strains showed robust morphology and functionality across the entire 14-day spheroid culture period (a total of 21 days in culture).

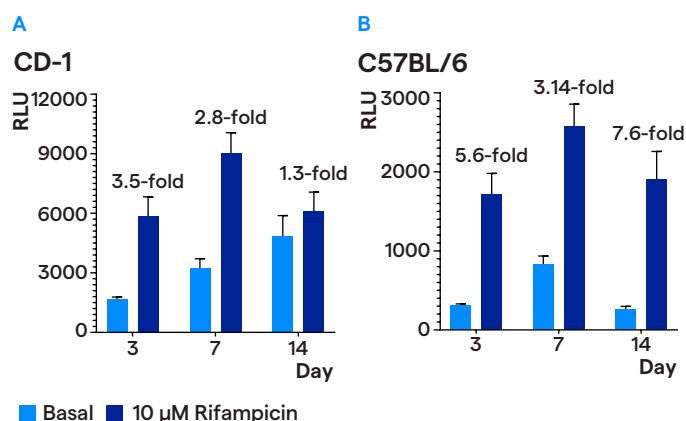


Figure 2. Cyp3a11 basal and induced activity levels in CD-1 and C57BL/6 mouse hepatocyte spheroids. Cells were induced with 10 μ M rifampicin for 72 hours. Data represents the mean of n=3 independent experiments, measuring 3 – 4 individual spheroids per time point.

DILI toxicity model with acetaminophen

Both mouse strains exhibited the expected dose-response to acetaminophen over time. Acetaminophen at higher doses elicits a chronic toxicity response in human hepatocytes whereby the EC_{50} value progressively decreases over time with repeated exposure.⁵ Consistent with this, our study showed that EC_{50} values for both mouse strains decreased significantly over time (Figure 3). Interestingly, there were also significant differences between strains, with strain C57BL/6 showing significantly greater toxicity at earlier timepoints compared with strain CD-1 (Figure 3). By day 14 of acetaminophen exposure (21 days total in culture), EC_{50} values for both strains had decreased to near the $10 \times C_{max}$ (10 times the maximum serum concentration) reported in humans,⁶ underscoring the predictive relevance of these mouse spheroid models for assessing acetaminophen-induced DILI.

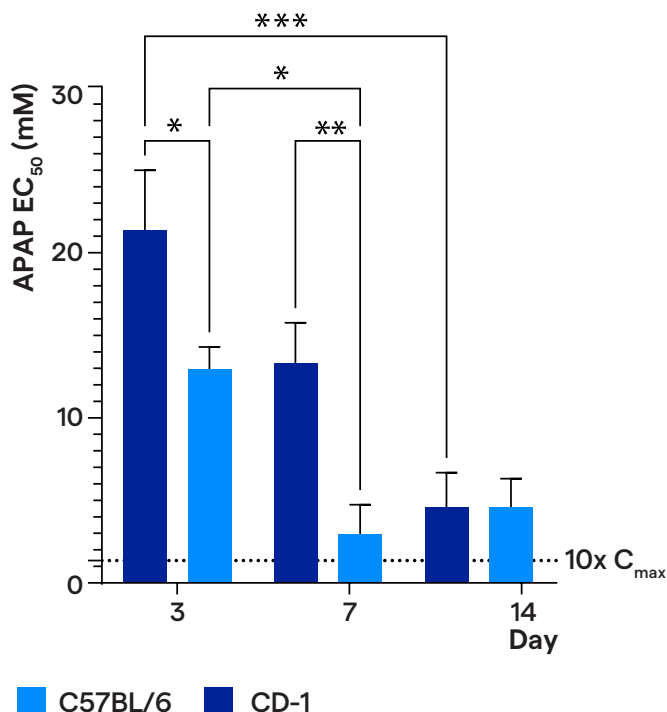


Figure 3. EC₅₀ values generated from dose-response curves (calculated as a function of ATP generation) following 3, 7, and 14 days of exposure to a range of acetaminophen concentrations. EC₅₀ represents the concentration at which a 50% effect was observed for each treatment duration in each mouse strain. The dashed line indicates 10x C_{max}, which is 10 times the maximum possible serum concentration of acetaminophen in humans.⁶ Data represents the mean of n= 3-4 replicate spheroids per time point. Statistical differences were determined by two-way ANOVA using Šidák's multiple comparisons test. *p<0.03, **p<0.002, ***p<0.0001.

Conclusions

This study demonstrates the development of a high-throughput 3D spheroid culture model for two mouse hepatocyte strains that maintains proper morphology and functionality over a 21-day culture period (14 days of spheroid culture). This long-term culture format enables the examination of both acute and chronic DILI toxicity, as demonstrated with the exemplar hepatotoxin acetaminophen, providing a physiologically relevant first step for screening compounds prior to *in vivo* animal testing, thereby reducing the overall number of live animals needed in the safety testing process.

References:

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