

## *In vitro* evaluation of hepatic function using a primary human hepatocyte 3D spheroid culture system

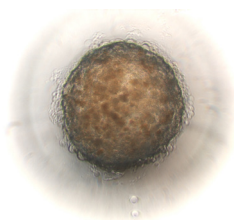
### Introduction

The conventional method of **culturing primary human hepatocytes** (PHH) in a 2-dimensional (2D) monolayer presents limitations in the study of hepatic biology, liver function, and drug-induced hepatotoxicity. Traditional 2D hepatocyte cultures dedifferentiate, resulting in the loss of specific hepatic function in approximately 5 days. We have developed and characterized a PHH 3-dimensional (3D) spheroid culture system that preserves hepatic function and promotes culture longevity.

Gibco™ PHH can easily be assembled into a 3D spheroid culture in 5 days using Thermo Scientific™ Nunclon™ Sphera™ low-attachment U-bottom 96-well microplates and **Gibco™ plating medium and plating supplements**. The 3D spheroid hepatocyte culture requires a significantly lower number of cells than its 2D counterpart, allowing this system to better support high-throughput assays. Moreover, the PHH in the 3D spheroid culture are functionally viable for at least 3 weeks, enabling long-term studies of hepatocyte function.

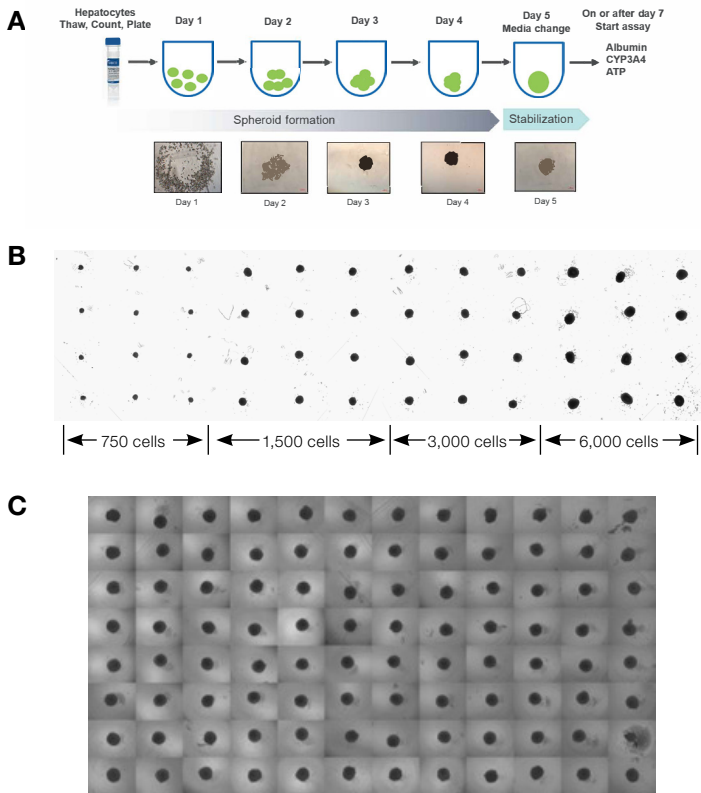
### Application 1: PHH 3D spheroid formation

1. Plating medium was made by adding Gibco™ Primary Hepatocyte Thawing and Plating Supplements (Cat. No. CM3000) to Williams E Medium (Cat. No. A1217601). The plating medium and Gibco™ Hepatocyte Thaw Medium (HTM) (Cat. No. CM7500) were warmed in a 37°C water bath.



2. Gibco™ 3D spheroid-qualified human hepatocytes (Cat. No. HMCPSQ) were thawed quickly in a 37°C water bath, and the contents of the tube were transferred to the tube of HTM.
3. The cells were centrifuged at 100 x g for 10 min, and the cell pellet was gently resuspended in 3 mL of the plating medium.
4. After counting the hepatocytes, 1,500 cells/well were plated in the Nunclon Sphera 96-well microplate (Cat. No. 174925).  
**Note:** 1,500 cells in 200 µL of medium (7,500 cells/mL) can be added to each well; or, after pre-wetting the plate with 100 µL of plating medium, 1,500 cells in 100 µL of medium (15,000 cells/mL) can be added to each well.
5. After plating the cells, the plate was centrifuged at 200 x g for 2 min to pellet cells to the bottom of the plate.
6. The seeded cells were placed in a 37°C incubator with 5% CO<sub>2</sub> and allowed to incubate for 3–5 days undisturbed before changing the medium. **Note:** It is important to place the plate in an incubator that is not being used with frequent opening for other cultures, and to gently close the incubator door to avoid disturbing the spheroid formation.

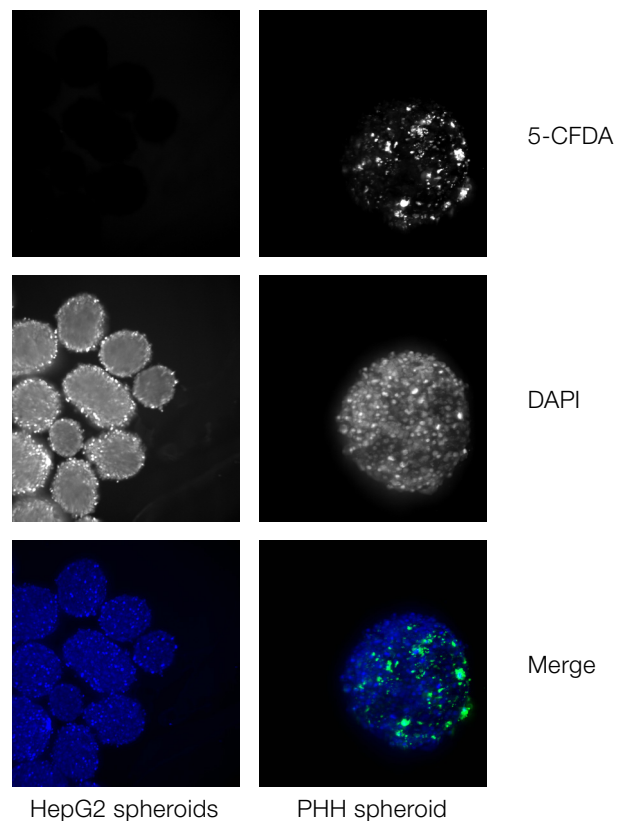
7. The spheroids formed within 5 days. No earlier than day 7, biochemical assays and characterization were performed. Hepatocyte maintenance medium (prepared by adding Hepatocyte Maintenance Supplement (Cat. No. CM4000) into Williams E Medium (Cat. No. A1217601)) was used for 50% medium exchanges every 48–72 hours (Figure 1). Medium exchanges can be completed using the Thermo Scientific™ Wellwash™ Versa Microplate Washer (Cat. No. 5165010).



**Figure 1. Workflow of assembly and characterization of primary hepatocytes in 3D spheroid culture.** (A) Spheroids were imaged in phase at 10x magnification. These images show spheroid formation by day 5 of culture. (B) Spheroid size is directly proportional to the number of cells seeded. Spheroids were imaged using the **Invitrogen™ EVOS™ FL Auto 2 Cell Imaging System** (Cat. No. AMAFD2000) at 4x. (C) Plating of hepatocyte spheroids in a Nunclon Sphera 96-well U-bottom microplate shows consistency in spheroid formation across the plate.

## Application 2: Analysis of formation of bile canaliculi in 3D hepatic spheroids

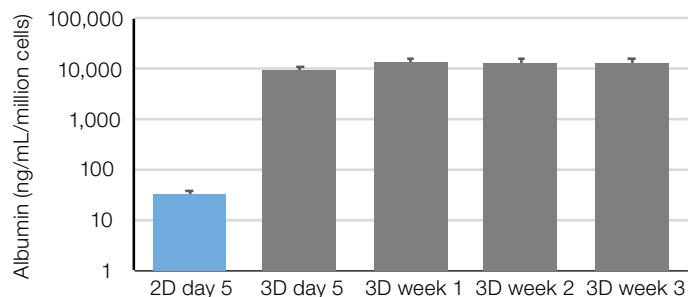
- Using Application 1, a 3D spheroid culture of hepatocytes was established.
- A working solution of 5  $\mu$ M 5-carboxyfluorescein diacetate (5-CFDA) (Cat. No. C1354), was prepared. 5-CFDA is used to visualize formation of bile canaliculi in the 3D spheroids. 5-CFDA permeates intact functional hepatocytes and is hydrolyzed to 5-carboxyfluorescein (5-CF), which is secreted out of the hepatocytes, accumulates in bile canaliculi, and exhibits strong fluorescence.
- During week 1, the medium was removed from the 3D hepatic spheroids, and they were treated with 100  $\mu$ L of 5  $\mu$ M 5-CFDA stock solution and incubated for 1 hr at 37°C.
- The wells were washed 3 times with hepatocyte maintenance medium, and the cells were imaged using transmission electron microscopy with GFP/FITC settings (Figure 2).



**Figure 2. Evaluation of formation of bile canaliculi in hepatic spheroids.** HepG2 spheroids during week 2 (left) and hepatic spheroids during week 1 (right) were stained with 5-CFDA and DAPI and imaged using the Thermo Scientific™ CellInsight™ CX7 platform at 10x magnification. Hepatic spheroids show clear formation of bile canaliculi in comparison to the HepG2 spheroids (used as the negative control).

### Application 3: Measurement of albumin produced by 2D or 3D spheroid hepatic cultures

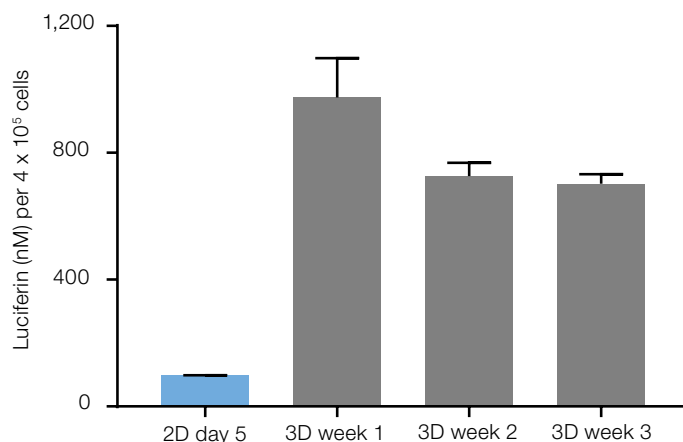
1. Using Application 1, a 3D spheroid culture of hepatocytes was established. Additionally, a 2D culture of hepatocytes was started.
2. On day 5 of the 2D hepatocyte culture and on various days of the 3D hepatic spheroid culture, 120  $\mu\text{L}$  of the cell culture medium from each of the wells of the 2D and 3D cultures were collected for analysis of albumin secretion.
3. The cell culture medium was centrifuged at  $3,000 \times g$  for 10 min, and the supernatant was collected for an ELISA assay using the Abcam Human Albumin ELISA Kit (Figure 3).



**Figure 3. Albumin secretion in 2D and 3D spheroid hepatic cultures.** The concentration of albumin secreted is normalized to the total number of cells per well.

### Application 4: Activity in 2D or 3D spheroid hepatic cultures

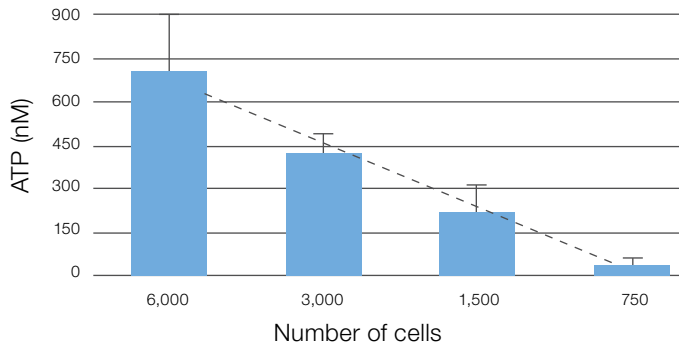
1. Using Application 1, a 3D spheroid culture of hepatocytes was established.
2. On day 5 of the 2D hepatocyte culture and on various days of the 3D spheroid culture, a total of 8 hepatic spheroids were transferred to a single well on a **Thermo Scientific™ Nunclon™ Delta™ 24-well plate (Cat. No. 142475)**.
3. The hepatocyte maintenance medium remaining in the 24-well plate after transfer of the spheroids was carefully removed by pipette and replenished with 500  $\mu\text{L}$  of fresh hepatocyte maintenance medium.
4. Activity of the liver enzyme CYP3A4 was measured on the day of the culture indicated in Figure 4, using the protocol for the Promega P450-Glo™ CYP3A4 Assay with Luciferin-IPA.



**Figure 4. CYP3A4 activity in 2D and 3D spheroid hepatic cultures.** CYP3A4 activity was measured using the Promega P450-Glo CYP3A4 Assay with Luciferin-IPA. CYP3A4 activity was found to be significantly higher in the 3D spheroids than in the 2D culture. The data presented are the mean  $\pm$  SEM ( $n = 3$  for the 2D culture,  $n = 8$  for the 3D spheroids).

### Application 5: ATP synthesis by 3D spheroid hepatic cultures

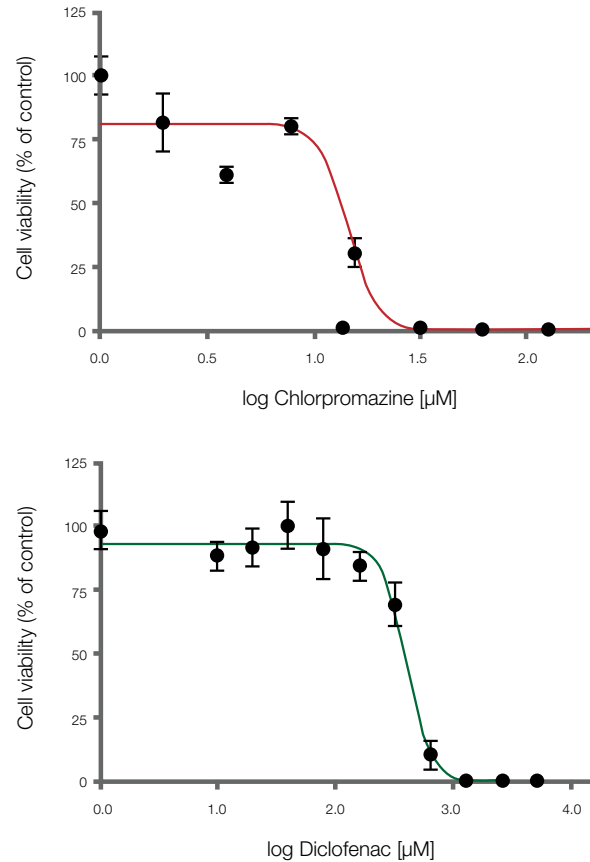
- Using Application 1, a 3D spheroid culture of hepatocytes was established.
- During week 1, ATP synthesis was measured in 3 replicates (Figure 5) using the Promega CellTiter-Glo™ 3D Cell Viability Assay.



**Figure 5. ATP synthesis by 3D spheroid hepatic cultures is proportional to the number of cells.** Using the CellTiter-Glo 3D Cell Viability Assay in 3 replicates, ATP synthesis by individual spheroids was measured during week 1.

### Application 6: Assay of drug-induced cytotoxicity using 3D spheroid hepatic cultures

- Using Application 1, a 3D spheroid culture of hepatocytes was established.
- During week 2 of culture, 3D spheroids were treated with variable levels of the antipsychotic drug chlorpromazine and the anti-inflammatory drug diclofenac, in 4 replicates.
- Cell viability was assayed 24 hours posttreatment using the protocol for the CellTiter-Glo 3D Cell Viability Assay. Nonlinear regression was performed for variable slope of log (inhibitor) vs. response using GraphPad Prism™ 7 Software (Figure 6). Table 1 shows that 2D and 3D spheroid hepatic cultures have comparable IC<sub>50</sub> values.



**Figure 6. Drug-induced cytotoxicity assayed using 3D spheroid hepatic cultures.**

**Table 1. IC<sub>50</sub> of drug-induced cytotoxicity for 2D hepatocytes and 3D spheroid hepatic cultures.**

Drug	IC <sub>50</sub> (2D culture)	IC <sub>50</sub> (3D culture)
Chlorpromazine	34 µM	14 µM
Diclofenac	331 µM	396 µM

### Conclusion

Collectively, these data confirm that cultures of 3D spheroid-qualified human hepatocytes have been characterized to show stable morphology, viability, and hepatocyte-specific functions for at least 3 weeks. We have demonstrated that our 3D spheroid-qualified hepatic cultures are functional, as indicated by formation of bile canaliculi as well as sustained albumin secretion. In comparing CYP3A4 activity on day 5 of 2D hepatic cultures and during week 1 of 3D spheroid hepatic cultures, we

have shown that 3D spheroid cultures have significantly higher activity (Figure 4). We also show that this 3D spheroid hepatic culture system can be used to analyze drug-induced cytotoxicity in hepatocytes. Ultimately, these data indicate that the reduced number of cells required for 3D spheroid formation as well as the sustained longevity of these cultures may better support high-throughput assays and long-term studies of hepatocyte functions.

### Ordering information

Product	Cat. No.
3D Spheroid-Qualified Primary Human Hepatocytes	HMCP SQ
Hepatocyte Thaw Medium	CM7500
Williams E Medium, no phenol red	A1217601
Primary Hepatocyte Thawing and Plating Supplements	CM3000
Primary Hepatocyte Maintenance Supplements	CM4000
Nunclon Sphera Microplates, low-attachment U-bottom 96-well	174925
Collagen I, Coated Plate, 24-well	A1142802
5-CFDA, AM (5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester)	C1354

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