In vitro evaluation of hepatic gene expression using a Gibco 3D spheroid culture system

Introduction

As research methods evolve, we must find innovative tools to create and understand more complex and physiologically relevant systems.

We have developed an easy-to-assemble, *in vitro* primary human hepatocyte (PHH) 3D spheroid model. In the previously released application note, we were able to prove that PHH 3D spheroid culture is a robust *in vitro* model that can survive 21 days in culture and improves hepatic function relative to 2D culture. To demonstrate this, we have analyzed the expression levels of various genes that play a role in human hepatocyte function, including ABC transporters and phase-1 P450 enzymes, in 3D hepatic spheroid cultures, and show that PHH 3D cultures can enable hepatic gene expression more efficiently than 2D hepatocyte cultures.

Application

PHH spheroids were formed using Gibco™ cryopreserved 3D spheroid–qualified human hepatocytes (Cat. No. HMCPSQ) and following the product user guide. After spheroid formation, total RNA was extracted using the Invitrogen™ PureLink™ RNA Mini Kit (Cat. No. 12183018A), RT-PCR was performed using the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Cat. No. 4374966), and qPCR using the Applied Biosystems™ TaqMan® Gene Expression Assays. The following protocol is designed to analyze a pool of multiple PHH spheroids (each spheroid contains approximately 1,500 cells) harvested from a 96-well Thermo Scientific™ Nunclon™ Sphera™ plate (Cat No. 174925).

Total RNA purification

1. Pool 16 spheroids into a microcentrifuge tube using wide-bore tips (to avoid shearing of spheroids) to transfer each spheroid along with 100 µL of medium into the pool. Once 16 spheroids have been pooled, remove as much of the medium as possible without aspirating off any spheroids. After removing the medium, add 1.0 mL of Invitrogen™ TRIzol™ Reagent to the tube containing the pooled spheroids.*
   Note: While 16 spheroids were pooled to generate data for this work, optimization is recommended to determine the appropriate number of spheroids to pool to obtain an ideal RNA yield for each lab.
2. To the cells in TRIzol Reagent, add 0.2 mL of chloroform per 1.0 mL of TRIzol Reagent and shake the tube vigorously by hand for 15 sec.
3. Incubate the sample at room temperature for 2–3 min, and centrifuge at 12,000 x *g* for 15 min at 4°C.
4. Transfer approximately 0.6 mL of the colorless upper phase containing the RNA to a fresh, RNase-free tube and add an equal volume of 100% ethanol to obtain a final ethanol concentration of 50%. Mix the sample well by vortexing.
5. Invert the tube to disperse any visible precipitate that may form after adding ethanol.

* Due to the nature of the 3D structures, we highly recommend the use of a strong denaturing or solubilizing approach such as using TRIzol Reagent. While other more gentle denaturing methods were evaluated, they yielded inconsistent results and low RNA recovery.

6. Add up to 0.7 mL of the sample to a spin cartridge (with a collection tube), and centrifuge at 12,000 x *g* for 15 sec at room temperature. The flow-through should be discarded, and the spin cartridge should be reinserted into the same collection tube.

7. Repeat steps 4–6 until the entire sample has been processed using the same spin cartridge.

8. Add 0.7 mL of wash buffer I to the spin cartridge, and centrifuge at 12,000 x *g* for 15 sec at room temperature.

9. Discard the flow-through and the collection tube, and insert the spin cartridge into a new collection tube.

10. Add 0.5 mL of wash buffer II with ethanol to the spin cartridge.

11. Centrifuge the tube at 12,000 x *g* for 15 sec at room temperature. Discard the flow-through and reinsert the spin cartridge into the same collection tube.

12. Repeat steps 10 and 11.

13. Centrifuge the spin cartridge and collection tube at 12,000 x *g* for 1 min at room temperature to dry the membrane with the bound RNA. Discard the collection tube and insert the spin cartridge into a recovery tube.

14. Add 0.03–0.1 mL of RNase-free water to the center of the spin cartridge, and incubate at room temperature for 1 min.

15. Centrifuge the spin cartridge with the recovery tube for 2 min at 12,000 x *g* at room temperature. Collect the purified total RNA from the recovery tube.

16. Quantify total RNA using a Thermo Scientific™ NanoDrop™ spectrophotometer and, if necessary, dilute the sample to 100 ng/µL using Invitrogen™ diethylpyrocarbonate (DEPC)-treated water.

cDNA preparation

1. Thaw the components of the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor on ice.

Table 1. RT master mix components.

Component	Per two wells	1 sample (25 wells)	Cat. No.
10X TaqMan RT Buffer	5 µL	50 µL	4319981
25X dNTP Mix	2 µL	20 µL	362271
10X Random Primers	5 µL	50 µL	4319979
MultiScribe Reverse Transcriptase (50 U/µL)	2.5 µL	25 µL	4319983
RNase Inhibitor (20 U/µL)	2.5 µL	25 µL	100021540
RNase-Free Water	8 µL	80 µL	R0603
Total	25 µL	250 µL	

2. Prepare the master mix by mixing the components as listed in Table 1.

3. Add 10 µL of 100 ng/µL total RNA to 10 µL of master mix.

4. Load the 20 µL mixture from step 3 into wells of a standard PCR plate.

5. Follow the thermal cycling conditions shown in Table 2.

Table 2. High-capacity cDNA thermal cycling program.

Step	Temperature	Time
1	25°C	10 min
2	37°C	120 min
3	85°C	5 min
4	4°C	Hold

qPCR preparation

1. Thaw the Applied Biosystems™ TaqMan® Fast Advanced Master Mix, probes, and nuclease-free water on ice.

2. Prepare the qPCR master mix on ice using the components listed in Table 3.

Table 3. qPCR master mix example.

Component	Volume per 20 µL reaction
TaqMan Fast Advanced Master Mix	10 µL
VIC probe	1 µL
FAM probe	1 µL
Nuclease-free water	6 µL
Total	18 µL

3. On ice, add 18 µL of master mix and 2 µL of cDNA to each well of an optical reaction plate.

4. Seal the plate with a cover and vortex for 5–10 sec, then briefly centrifuge.

5. Follow the qPCR program shown in Table 4.

Table 4. qPCR program.

Step	No. of cycles	Temperature	Time
Initial denaturation	1	95°C	20 sec
Amplification	40	95°C	3 sec
		60°C	20 sec

Results and summary

As we migrate to working with more phenotypically stable PHH 3D cultures, it is important to understand how closely gene expression mimics *in vivo* hepatic phenotypes and, moreover, how levels of gene expression in 3D cultures compare to those in traditional 2D hepatocyte cultures. Here we have validated a method to easily purify and analyze RNA samples from PHH 3D cultures out to 28 days in culture. Data generated in this study show that across multiple single-donor lots, levels of albumin, CYP3A4, CYP2D6, and MDR1 mRNA are consistently higher in 3D spheroids than in 2D culture (Figure 1). Average gene expression in 3D spheroids ranges from 2- to 36-fold higher than in traditional 2D cultures. While there is variability from donor to donor and among the genes tested, in general the fold change relative to 2D cultures peaks between weeks 1 and 2. These data indicate that across a panel of 4 genes, stable PHH 3D cultures enable higher levels of gene expression than traditional 2D hepatocyte cultures. Moreover, data shown here further validate the longevity advantage of PHH 3D cultures over traditional 2D cultures, as sustained gene expression was observed for multiple weeks in 3D cultures.

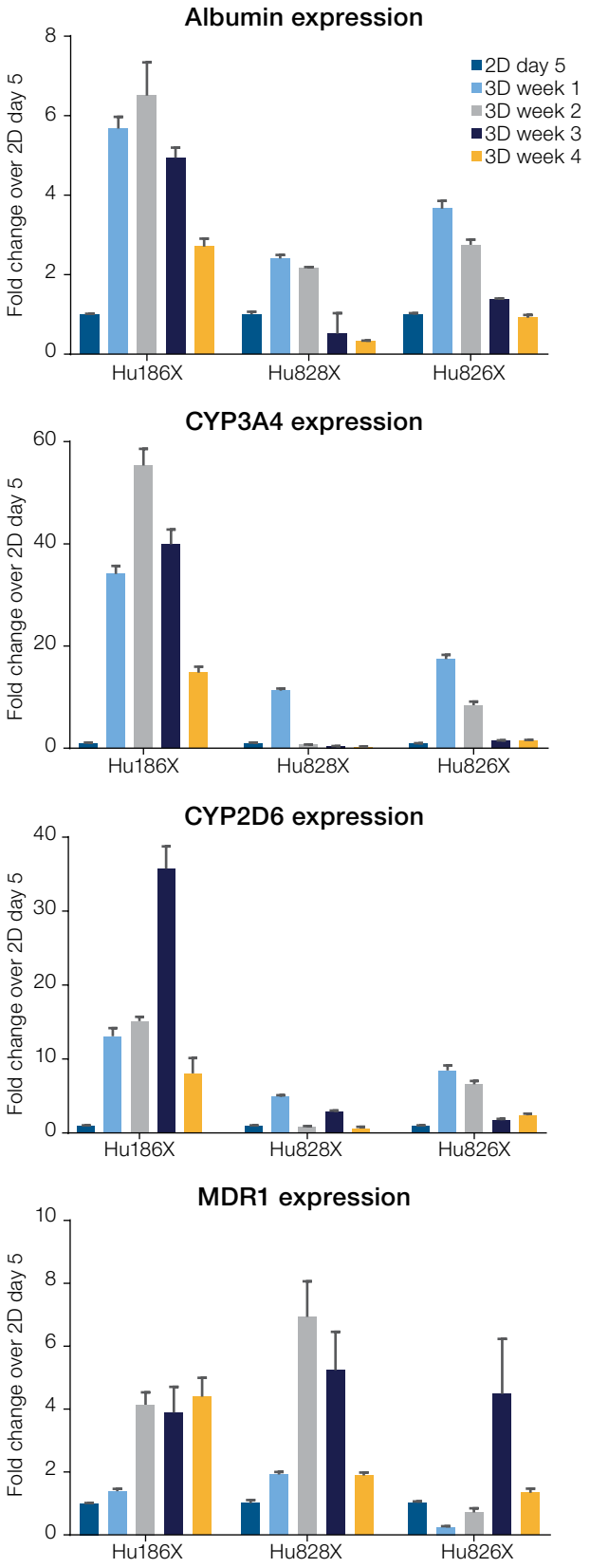

Figure 1. Time course evaluation of hepatic gene expression shows elevation in spheroid cultures. qPCR results for albumin, CYP3A4, CYP2D6, and MDR1 mRNA levels of 3 individual lots of Gibco 3D spheroid–qualified human hepatocytes. Gene expression levels of 3D cultures at different time points were normalized to day 5 of 2D culture. Each 3D spheroid sample contained a pool of 16 spheroids. Results are mean ± SEM, n = 3 spheroid pool samples.

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Ordering information

Product	Cat. No.
PureLink RNA Mini Kit	12183018A
3D Spheroid–Qualified Human Hepatocytes	HMCPSQ
High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor	4374966
Nunclon Sphera Plate	174925
NanoDrop One/One Microvolume UV-Vis Spectrophotometer	ND-One-W
DEPC-Treated Water	4387937
TaqMan Fast Advanced Master Mix	4444556
10X TaqMan RT Buffer	4319981
25X dNTP Mix	362271
10X Random Primers	4319979
MultiScribe Reverse Transcriptase (50 U/µL)	4319983
RNase Inhibitor (20 U/µL)	100021540
RNase-Free Water	R0603

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