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

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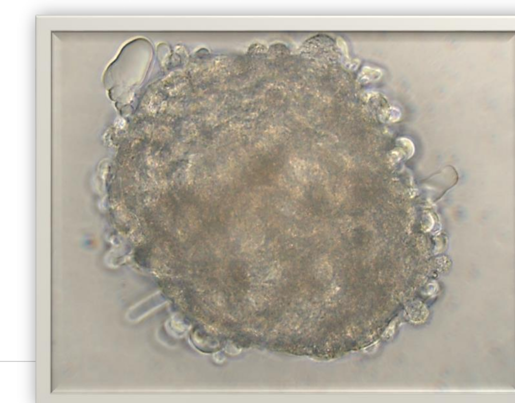



## ABSTRACT

Recent evidence suggests that culturing primary hepatocytes in 3-dimensional (3D) hepatic spheroids offers major advantages in providing longevity and increased physiological relevance in modeling liver metabolism. Dog (Beagle), Mouse (CD-1), and Rat (Sprague Dawley) hepatocytes from Thermo Fisher Scientific were plated for spheroid qualification. 3D hepatic spheroids were characterized in weeks 1-4 in which the following were assessed: cell viability (as indicated by ATP synthesis), albumin production, phase I metabolic activity, and gene expression. Our data indicate that primary Dog and Rodent hepatocytes formed 3D spheroids by Day 5, and moreover were viable and functional for up to 4 weeks. In conclusion, our data indicate that animal 3D spheroid cultures are an accurate and sustainable *in vitro* model of hepatocyte function that maintain hepatic functions for a longer period of time in comparison with traditional 2D cultures.


## INTRODUCTION

Conventional methods of culturing primary hepatocytes in 2-dimensional monolayer (2D) present limitations for the study of hepatic biology, liver function, and drug induced hepatotoxicity. Traditional 2D hepatocyte cultures rapidly de-differentiate resulting in the loss of hepatic specific function in approximately 5 days. While there are data to support that culturing primary human hepatic spheroids is a sustainable and robust *in vitro* model, limited information is available on the use of 3D animal spheroids in drug discovery. It is our hypothesis that growing animal hepatocytes in 3D spheroid cultures will more accurately reflect *in vivo* liver biology and maintain the liver functions for a longer period of time in comparison with the traditional 2D culture. Here we describe the incorporation of primary Dog and Rodent hepatocytes into 3D-culture systems that can be readily scaled for screening applications and studies where long term viability is important.

## MATERIALS AND METHODS

**Media and Reagents:**

Plateable Dog (Beagle) ( cat# DGCP10), Plateable Mouse (CD-1) Cryopreserved Hepatocytes (cat# MSCP10 ); Plateable Rat (Sprague-Dawley) Cryopreserved Hepatocytes (cat# RTCP10); Hepatocyte Plating medium – Supplemented Williams Media E, WEM (Gibco™ cat# A1217601)
Hepatocyte Maintenance Medium – For Dog: Supplemented WEM (Gibco™ cat# A1217601) For Rodent: Supplemented DMEM Hepatocyte Thaw Medium - HTM (Gibco™ cat# CM7500)
3D Plates - Nunclon™ Sphera™ low attachment U-bottom 96-well Microplates (cat# 174925)
2D Collagen Plates (controls) – Gibco™ Collagen Coated 24-well plates (cat# A1142802)

**Method**

1. Cryopreserved Rat and Mouse Primary Hepatocyte (Gibco™) vials were thawed quickly in a 37°C water bath. Upon thawing the cells were promptly transferred into a 50 mL centrifuge tube containing HTM.
2. The tubes were centrifuged to pellet the cells. After centrifugation the supernatant was discarded.
3. The cell pellet was gently re-suspended in 1 mL hepatocyte plating media, followed by additional 3 ml. Cell counting was performed with Trypan blue using hemocytometer.
4. Appropriate volume of cell suspension was prepared to contain desired number of cells. Using a multichannel pipette 200uL of the cell suspension was added into individual well of Nunclon™ Sphera™ low attachment U-bottom 96-well Microplates. Cell numbers between 375 and 6000 cells/well were used.
5. The Sphera™ microplates were centrifuged at 200xg for 2 minutes to allow cells to group at the bottom of the wells. **NOTE:** Dog cells were centrifuged at 500g for 4 minutes.
6. The Sphera™ Microplates were transferred to an incubator (37° C, 5% $CO_2$, humidified) and allowed to sit undisturbed in Plating Media for 5 days. **NOTE:** Dog cells were centrifuged at 125g for 2 minutes on Days 1 and 2. (Figure 1)
7. By 5-days the hepatocytes formed spheroid with compact spherical mass of cells (Figure 1 and 2). On day 5, upon confirmation of spheroid formation, 50% the plating media was exchanged with hepatocyte maintenance media.

## RESULTS

**Figure 1. Work Flow of assembly and characterization of primary hepatocyte into 3D-spheroid**

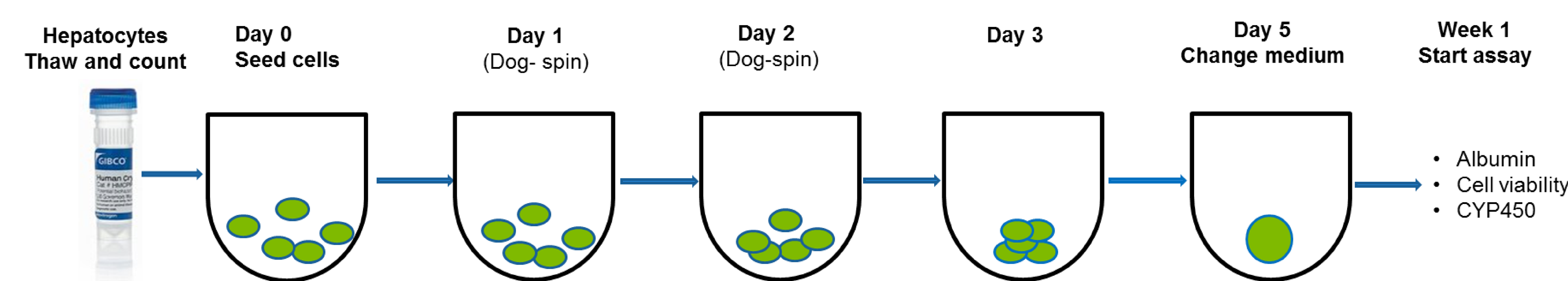
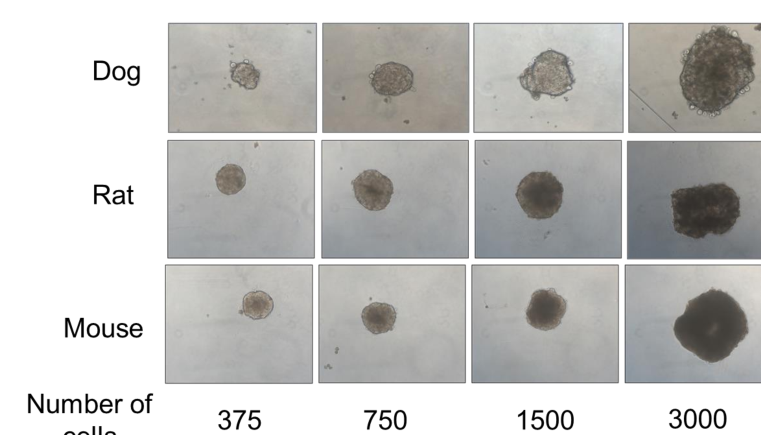
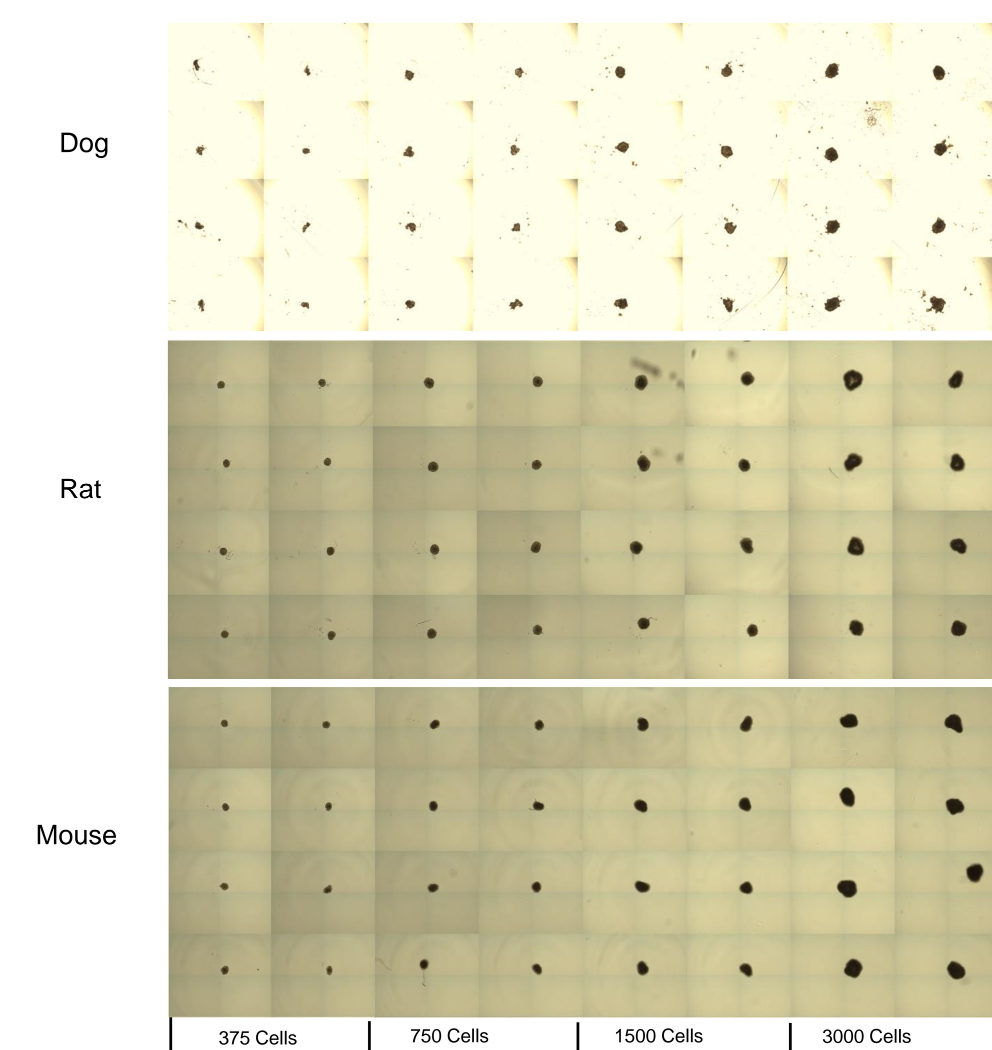



**Figure 2. Dog and Rodent Hepatic Spheroids using Variable Cell numbers**



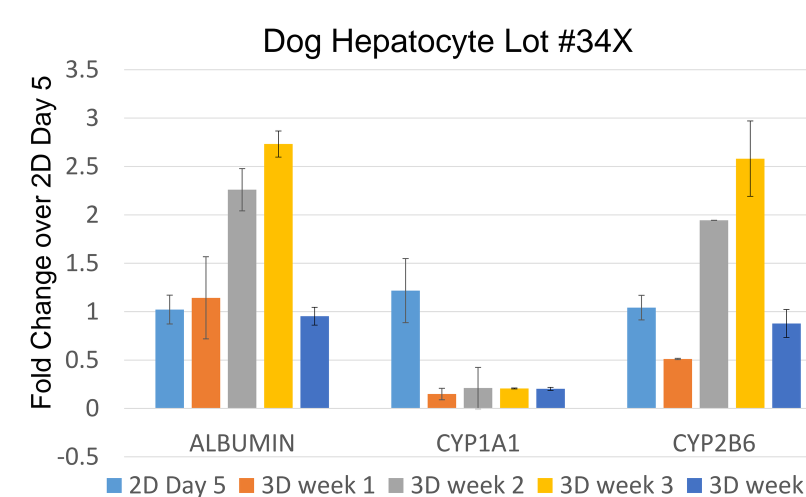

Dog and Rodent primary hepatocytes were seeded at different cell densities. During week 1 the spheroids were imaged using bright field microscopy at 10x optical zoom. Representative images from one lot per species are presented. Data not shown: Plating media supplemented with 20% serum was tested in the formation of dog spheroids, however this change did not impact the workflow outlined in Figure 1. or the results shown above.

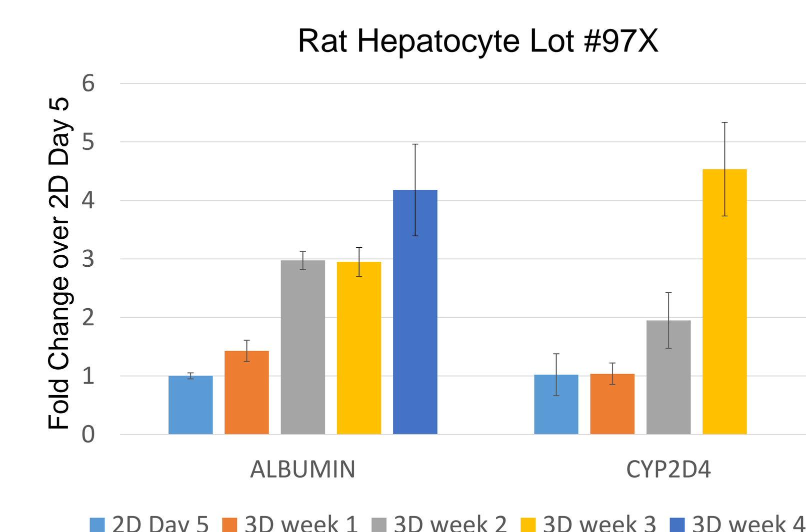
**Figure 3. Optimization of Hepatic Spheroids: Varying Cells/Well**

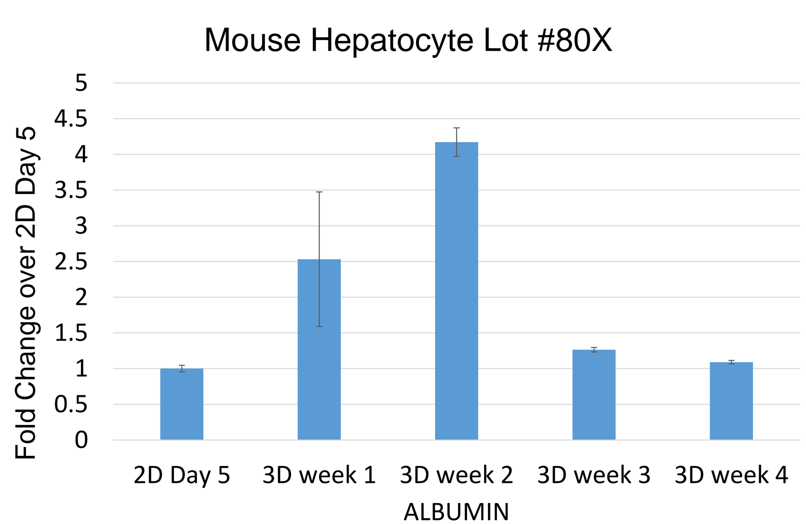



Primary Hepatocytes were seeded in Nunclon™ Sphera™ low attachment U-bottom 96-well Microplates in variable cell numbers.

**Figure 4. Comparison of Gene Expression between 2D and 3D cultures**

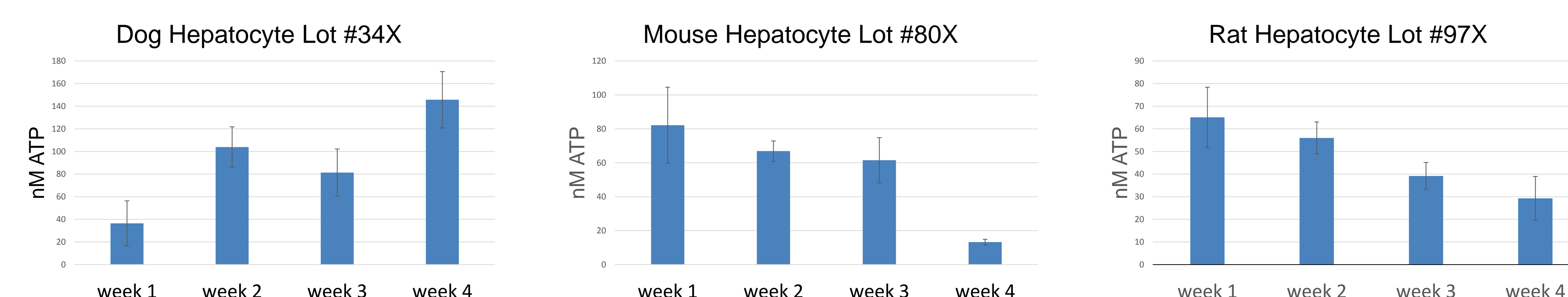


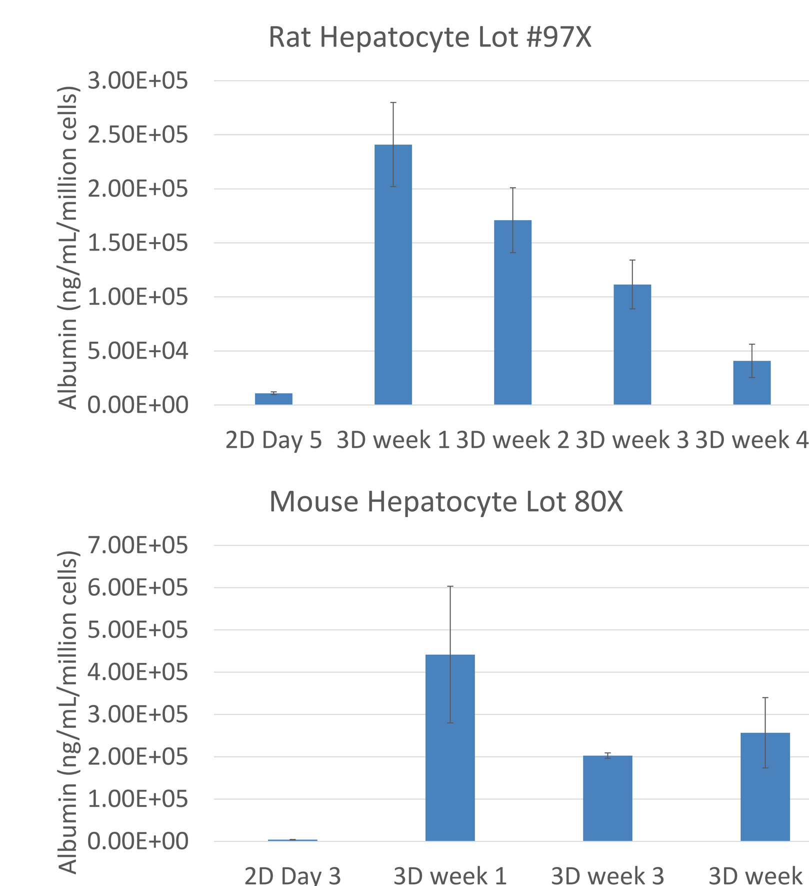

Gene expression levels were measured by qRT-PCR to compare the levels of gene expression between day 5 of 2D culture and various days of 3D culture.

**Figure 5. ATP synthesis by 3D spheroids**



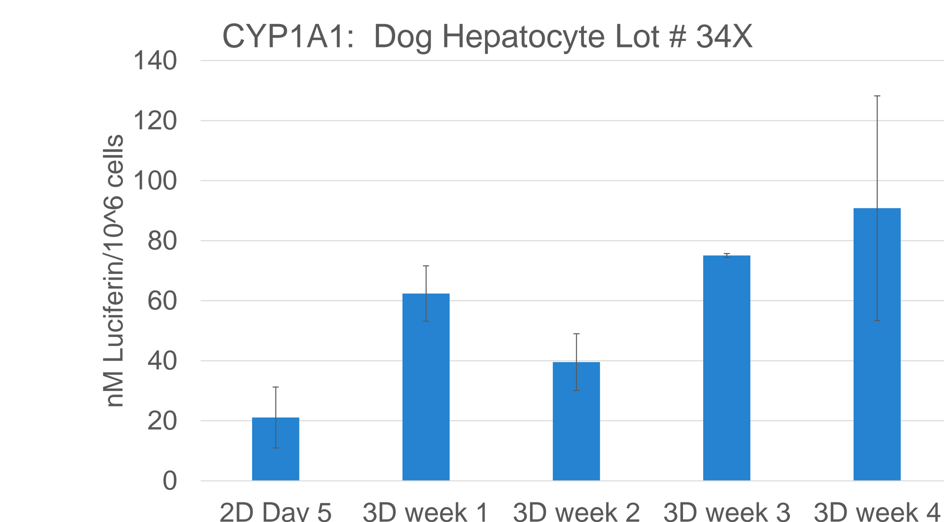

In weeks 1-4 of the spheroid culture the viability of the spheroids was assessed by measuring the ATP levels of 3 individual spheroids using a CellTiter-Glo® 3D cell viability assay (Promega). Results are mean +/- SEM, n = 3. Data not shown: modified parameter in ATP assay for Mouse and Rat Hepatocyte lots #80X and #97X with additional agitation, however this modification did not impact results shown above.

**Figure 6. Albumin Production**




The concentration of albumin secreted was measured by an ELISA, (Abcam) and normalized to the total number of cells per well. Results are mean +/- SEM, n = 3.

**Figure 7. CYP450 Activity**




In weeks 1-4, 8 spheroids were pooled, CYP1A1 activity was measured using the P450 ProGlo assay (Promega). Results are mean +/- SEM, n = 2.

## CONCLUSIONS

- Gibco™ Primary Dog, Mouse and Rat Hepatocytes can easily be assembled into a 3D culture in 5 days using Nunclon™ Sphera™ low attachment U-bottom 96-well Microplates, Gibco™ plating media & plating supplements and our recommended protocol.
- All animal hepatocyte lots tested for spheroid formation have formed spheroids. (2 lots of dog primary hepatocytes, 4 lots of mouse primary hepatocytes, and 3 lots of rat primary hepatocytes formed 3D-spheroids.)
- Dog spheroid formation requires additional spin steps on Days 1 and 2 for optimal results- these may be useful additional steps for lots refractory to spheroid generation.
- From data not shown- we've found multiple assays ( CYP450 assays, ATP, others) or sample prep workflows designed for 2D that appear to require modification if they are to be useful for 3D applications.

## FUTURE DIRECTIONS

- Explore alternative methods to assess viability and to measure CYP450 activity in animal spheroids.

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