**Primary hepatocytes in RAFT™ 3D Cell Culture System –
a model for hepatocyte toxicity studies**
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Lonza

Pharma & Biotech

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Introduction

Hepatocytes are the primary cell type of the liver and function to provide the majority of the detoxification in the body. In the pharmaceutical industry, there is an increasing demand for hepatocyte-based models that mimic human *in vivo* toxicity.[1, 2] Typical *in vitro* drug toxicity screening has incorporated hepatocytes grown in a two-dimensional (2D) format, cultured between collagen-coated tissue culture plastic and Matrigel® ("Sandwich Culture"). However, literature has reported that although primary hepatocytes briefly maintain some functional capabilities in 2D culture, such as the production of drug metabolizing enzymes, they rapidly lose their morphology along with the majority of liver-specific functions.[3-5] A model demonstrating stronger responses and showing more stable maintenance of liver functions is desirable when assays that need more than 4 or 5 days in culture are required. The potential of three-dimensional (3D) models to mimic the *in vivo* cellular environment more closely have made them attractive alternatives to 2D culture systems.

We offer a novel RAFT™ (Real Architecture for Tissue) 3D Cell Culture System which allows the creation of tissue-like structures. The 3D matrix of the type 1 collagen-based RAFT™ 3D Cell Culture System provides a more natural cell culture environment, supporting primary cell phenotypes for longer durations than standard 2D culture systems. This feature of the RAFT™ 3D Cell Culture System potentially enables better predictive value for toxicity and metabolism studies when long-term studies are beneficial.

In this White Paper, we report a 3D hepatocyte model constructed using the RAFT™ 3D Cell Culture System (Figure 1) and characterization. The model consists of primary hepatocytes embedded in compressed RAFT™ Hydrogel. We compare cell viability, cell morphology and maintenance of Cytochrome P450 (CYP) activity of primary human hepatocytes grown in the traditional Sandwich Culture with that of cells cultured in RAFT™ 3D Cell Culture. Our results show that the RAFT™ 3D Cell Culture System improves the long-term maintenance of hepatocyte metabolic functions.

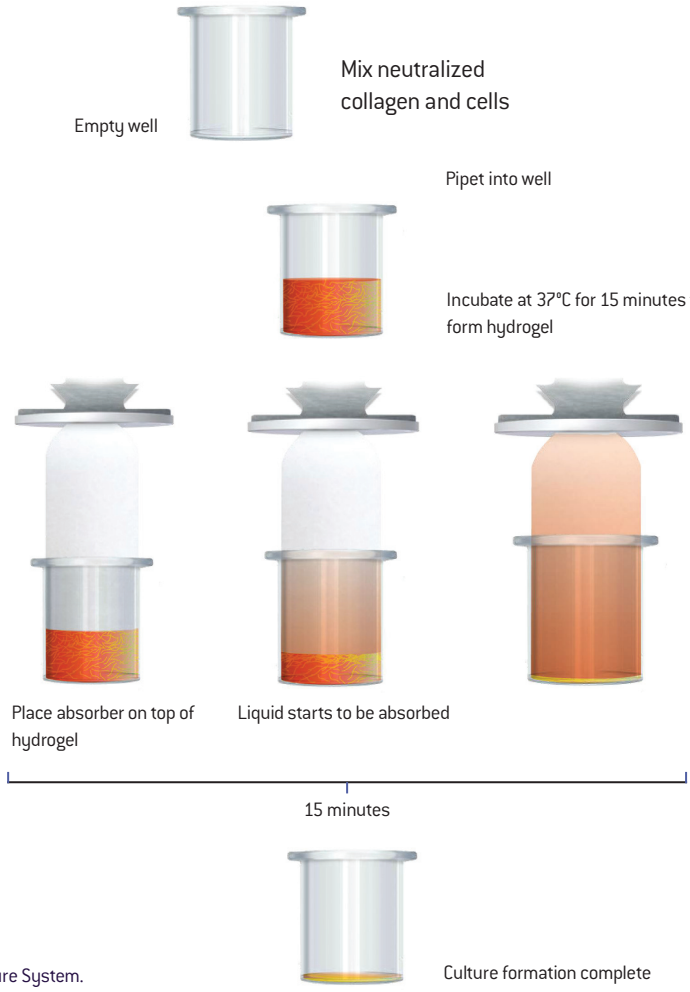


Figure 1
Process overview for RAFT™ 3D Cell Culture System.

Materials and methods

- Cryopreserved human primary hepatocytes (Lonza, cat. no. HUCP1 or HUCPG)
- Collagen coated 96-well plates (Corning, cat. no. 354407)
- 96-well tissue culture treated plates (Greiner, cat. no. 655180)
- RAFT™ Reagent Kit for 3D Culture (Lonza, cat. no. 016-0R94)
- RAFT™ Absorbers (Lonza, cat. no. 016-0R92)
- Matrigel® Matrix (Corning, cat. no. 354234)
- CellTiter-Glo® 3D Viability Assay – CTG (Promega, cat. no. G9681)
- alamarBlue® Cell Viability Reagent (Thermo Fisher Scientific, cat. no. DAL1025)
- P450-Glo™ CYP1A2 Assay (Promega, cat. no. V8421)
- P450-Glo™ CYP2B6 Assay (Promega, cat. no. V8321)
- P450-Glo™ CYP3A4 Assay (Promega, cat. no. V9001)
- Methanol (Sigma, cat. no. 179337)
- Normal Goat Serum – NGS (Sigma, cat. no. G6767)
- Anti-ZO-1 antibody (Thermo Fisher Scientific, cat. no. 33-9100)
- DAPI (Thermo Fisher Scientific, cat. no. D1306)
- Alexa Fluor 555 (Thermo Fisher Scientific, cat. no. A21425)
- Mouse IgG1 (Dako, cat. no. X0931)
- Phosphate Buffered Saline – PBS (Lonza, cat. no. 17-516Q)
- Glycerol (Thermo Fisher Scientific, cat. no. BP229-1)
- Rifampicin (Sigma, cat no. R3501)
- Phenacetin (Sigma, cat. no. 77440)
- Bupropion (Sigma, cat. no. B102)
- Testosterone (Sigma, cat. no. T1500)
- Salicylamide (MP Biomedicals, cat. no. 102835)
- Human Cryopreserved Hepatocyte Thawing Medium (Lonza, cat. no. MCHT50)
- HBM™ Hepatocyte Basal Medium (Lonza, cat. no. CC-3199)
- HCM™ SingleQuots™ Kit – Hepatocyte Culture Medium (Lonza, cat. no. CC-4182)
- Plating Medium (Lonza, cat. no. MP100-1)
- Plating Medium Supplement (Lonza, cat. no. MP100-2)
- Albumin Elisa Kit (Thermo Fisher Scientific, cat. no. EHALB)
- Urea Assay Kit (Abnova, cat. no. KA1652)

Cell culture

Preparation of HCM™ Hepatocyte Culture Medium

Hepatocyte Culture Medium SingleQuots™ were thawed overnight at 4°C. In a Biosafety Cabinet (BSC) thawed SingleQuots™ were added one at a time using a separate pipet for each growth factor, to a 500 mL bottle of HBM™ Medium, and swirled to mix thoroughly.

Human hepatocytes

Cryopreserved hepatocytes were thawed at 37°C. Each ampoule was diluted in 50 mL Human Hepatocyte Thawing Medium and centrifuged at 100 x g for 8 minutes at room temperature. After carefully aspirating most of the supernatant and leaving approximately 100 µL of Human Hepatocyte Thawing Medium over the pellet, the pellet was resuspended by tapping the tube against the palm of the hand. For counting, a small aliquot of concentrated cell suspension was diluted and counted using the trypan blue exclusion method. Because many hepatocytes contain two or more nuclei[6], it is strongly recommended that these cells be counted via trypan blue exclusion methods rather than procedures utilizing nuclear staining methodology. After counting cells, suspension was diluted with complete HCM™ Medium. A cell seeding density of 45,000–65,000 cells per 96-well is optimal. For a seeding density of 47,000 cells per well, for example, the cell concentration was adjusted to 9.4 x 10^5/mL (Sandwich Culture) or 1.9 x 10^7/mL (RAFT™ 3D Cell Culture).

Sandwich Culture

To the wells of collagen coated 96-well plates, 50 µL complete HCM™ Medium was added followed by 50 µL of cell suspension, for example, at cell concentration of 9.4 x 10^5/mL to give a final cell density 47,000 cells per well. Cells were allowed to settle by leaving the plates undisturbed in the BSC for 5 minutes. Plates were then placed in a 37°C, 5% CO_2 incubator for 2 hours. To remove non-viable cells, the medium was carefully aspirated and replaced with fresh HCM™ Medium. Cultures were incubated for an additional 2 hours. The medium was aspirated and the plates were overlaid with 0.3 mg/mL Matrigel® in HCM™ Medium. Cultures were refed daily with 100 µL HCM™ Medium per well.

RAFT™ 3D Cell Culture

Manufacturer's instructions for construction of RAFT™ 3D Cell Culture was followed except one quarter of the recommended volume (60 µL cell seeded collagen hydrogel per well of a 96-well plate, rather than the standard 240 µL) of hydrogel was used (see Lonza Tech Note: Construction of a 3D hepatocyte model using the RAFT™ 3D Cell Culture System). Final cell density in RAFT™ 3D Cell Cultures was 45,000–65,000 cells per well. Cultures were refed daily with 100 µL warm HCM™ Medium per well.

Assay kits

Assay kits (alamarBlue® Cell Viability Reagent, P450-Glo® CYP1A2, CYP2B6 and CYP3A4) were used as per the manufacturers' instructions. Cells were incubated with the reagent for 80 minutes at 37°C prior to assay evaluation.

Immunofluorescence

Sandwich Culture and RAFT™ 3D Cell Culture were fixed with ice cold Methanol for 10 minutes and rinsed twice with PBS. Cultures were blocked with 10% NGS in PBS for 40 minutes at room temperature. The blocked cultures were incubated with 50 µL per well of 5 µg/mL ZO-1 antibody or mouse IgG1 at 4°C overnight and then rinsed three times with PBS. Cultures were incubated with 50 µL per well of a 1:200 dilution (10 µg/mL) of Alexa Fluor 555 in 1% NGS at 37°C for 30 minutes. After rinsing three times with PBS, cultures were incubated with 50 µL per well of 100 µM DAPI in PBS at room temperature for 8 minutes. Cultures were rinsed three times with PBS and 50% Glycerol in PBS was placed in each well.

CYP450 activity quantitation

Inducers and substrates stock solutions at 1000x were prepared in DMSO and stored at -20°C. For CYP3A4, on the day of induction, the culture medium was aspirated and 100 µL HCM™ Medium containing either 10 µM rifampicin or 1:1000 DMSO (Control) was applied for 72 hours with daily refeeds. Cultures were incubated in a 37°C, 5% CO_2 incubator.

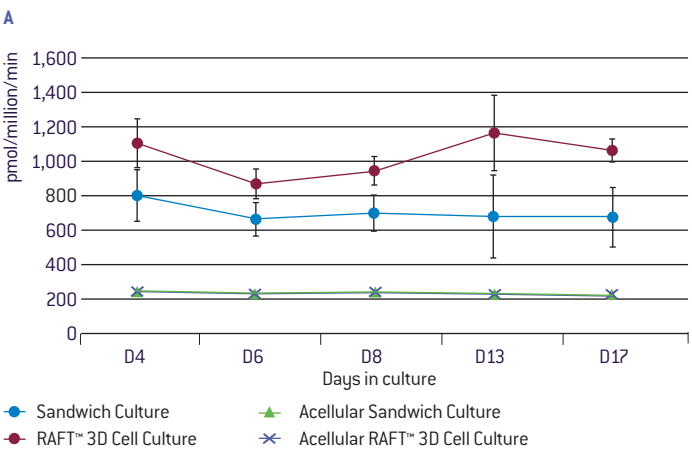
After the cells were exposed to inducer (or DMSO control) for 72 hours, they were rinsed once with warm HCM™ Medium. The corresponding substrates phenacetin (100 µM, CYP1A2), bupropion (250 µM, CYP 2B6) or testosterone (200 µM, CYP3A4) were applied to induced and non-induced (DMSO control) wells for 15 minutes (phenacetin or testosterone) or 20 minutes (bupropion) in 100 µL per well HCM™ Medium. The cultures were placed in the incubator on an orbital shaker at 95 rpm. At the end of the incubation period, the culture supernatants were collected. Basal CYP activities of non-induced cells were determined by evaluating supernatants of DMSO control wells. Metabolite quantification was determined by Liquid Chromatography Mass Spectrometry (LCMS).

Results and discussion

Cell viability of hepatocytes in RAFT™ 3D Cell Culture and Sandwich Culture can be determined using the alamarBlue® Reagent

The alamarBlue® Cell Viability Reagent was used to determine the viability of human hepatocytes in RAFT™ 3D Cell Culture and Sandwich Culture (Figure 2A). The alamarBlue® Reagent uses resazurin, a cell permeable, blue, non-fluorescent compound, as the active ingredient. Resazurin is reduced to resorufin, a red and highly fluorescent compound; within the cytoplasm of the cell.[7] Viable cells continuously convert resazurin to resorufin, and thereby increase the fluorescence of the culture medium. The data shown in Figure 2A illustrates that, using this reagent, human hepatocytes maintained a higher viability in RAFT™ 3D Cell Culture than in Sandwich Culture.

The morphology of hepatocytes embedded in the collagen matrix of RAFT™ 3D Cell Culture is shown in Figure 2B. The individual cells show a rounded morphology. This may be contrasted to the cuboidal morphology of hepatocytes grown in Sandwich Culture.[8]



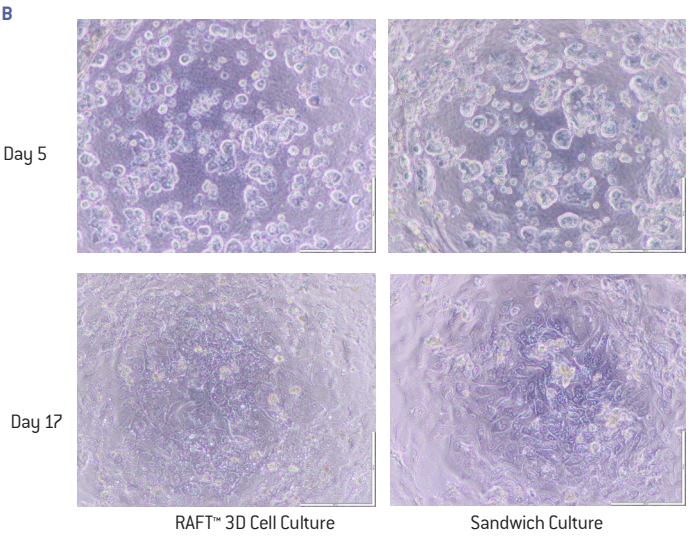


Figure 2

Cryopreserved human hepatocytes exhibit higher viability in RAFT™ 3D Cell Culture than in Sandwich Culture. (A) The viability of cryopreserved human hepatocytes cultured in RAFT™ 3D Cell Culture and Sandwich Culture were compared using the alamar Blue® Cell Viability Reagent. Cells were plated at 47,000 cells per well in each respective model. Error bars represent standard deviations between triplicates of one experiment. The survival of human hepatocytes is more robust in RAFT™ 3D Cell Culture than in traditional Sandwich Culture. (B) The morphology of primary human hepatocytes in RAFT™ 3D Cell Culture and Sandwich Culture. In RAFT™ 3D Cell Culture, the cells exhibit a rounded morphology within the collagen matrix.

RAFT™ 3D Cell Cultures of human hepatocytes maintain high viability when plated in serum containing medium or in serum-free medium

The RAFT™ 3D Cell Cultures of human hepatocytes were constructed and maintained in HCM™ Medium, a serum-free medium. To confirm that the absence of serum had no adverse effects on the cultures, RAFT™ 3D Cell Culture Hepatocytes were tested for morphology and viability at 2, 4, 6, 24 and 48 hours post plating in Plating Medium containing serum compared to that in HCM™ Medium followed by exchange of media to HCM™ Medium. As shown in Figure 3, for 48 hours post plating, serum-containing Plating Medium was comparable to HCM™ Medium. Similar results were obtained for other time points (data not shown). Sandwich Cultures were tested for 2 and 4 hours post plating in Plating Medium and were comparable for morphology and viability to hepatocytes plated in HCM™ Medium (data not shown).

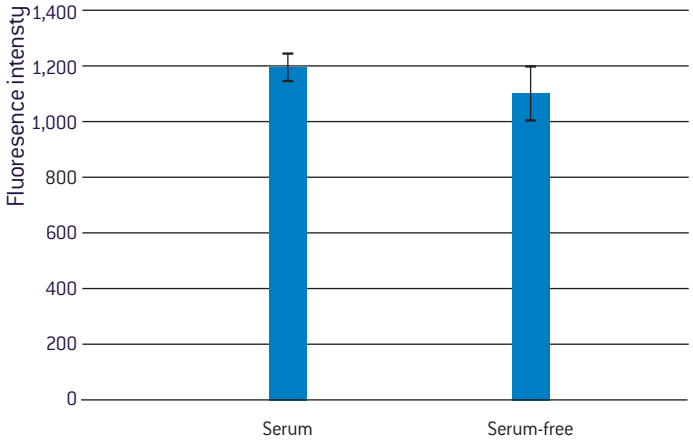


Figure 3

Human hepatocytes exhibit similar viabilities when plated in serum-containing medium and in serum-free medium. Hepatocytes were plated at 65,000 cells per well in either serum-containing medium (Plating Medium) or in serum-free medium (HCM™ Medium). Cultures were refed daily. At 48 hours post plating, cells in Plating Medium were refed with HCM™ Medium. At 72 hours post plating, the relative cell viability was determined using the alamarBlue® Reagent. The data shows that cell viabilities in both conditions are similar.

Tight junctions are formed in human hepatocytes in RAFT™ 3D Cell Culture and in Sandwich Culture

Tight junctions are of physiological importance to hepatocytes, not only do they modulate the passage of small molecules and ions between cells[9] but they also function to keep bile in the canaliculi and out of the blood circulation.[10] Immunofluorescence staining of human hepatocytes with the ZO-1 antibody shows that tight junctions are formed in both RAFT™ 3D Cell Cultures (Figures 4 A, 4 B) and Sandwich Cultures (Figures 4C, 4D).

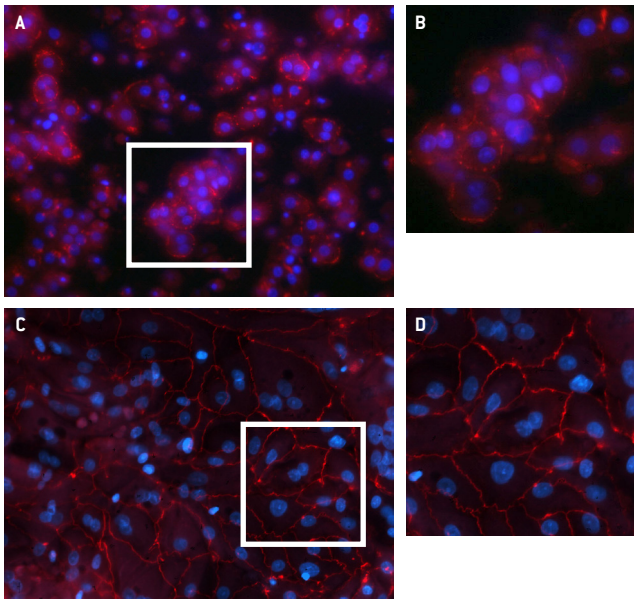


Figure 4

Cryopreserved human hepatocytes form tight junctions in RAFT™ 3D Cell Culture. Cryopreserved human hepatocytes were plated at 47,000 cells per well in the RAFT™ 3D Cell Culture and cultured for 4 days (RAFT™ 3D Cell Culture: A, B) or 6 days (Sandwich Culture: C, D). Methanol fixed cultures were stained for tight junctions with the ZO-1 antibody (red) and counter stained with DAPI (blue). (A) ZO-1 stained RAFT™ 3D Cell Culture 20x. (C) ZO-1 stained Sandwich Culture 20x. Insets (B, D) are enlarged to show detail.

Albumin secretion by human hepatocytes is similar in the RAFT™ 3D Cell Culture and in Sandwich Culture

The maintenance of serum albumin concentrations in the blood is an important function of hepatocytes. Albumin secretion by these cells maintains the osmotic pressure of the body whereby fluid is pulled into the capillaries. Reduction in serum albumin results in edema and impaired kidney function.[11] We compared the albumin concentration in the culture medium of hepatocytes grown in the RAFT™ 3D Cell Culture and in Sandwich Culture using a human albumin ELISA kit (Figure 5). The results show similar concentrations of albumin in conditioned medium of human hepatocytes grown in RAFT™ 3D Cell Culture and in Sandwich Culture.

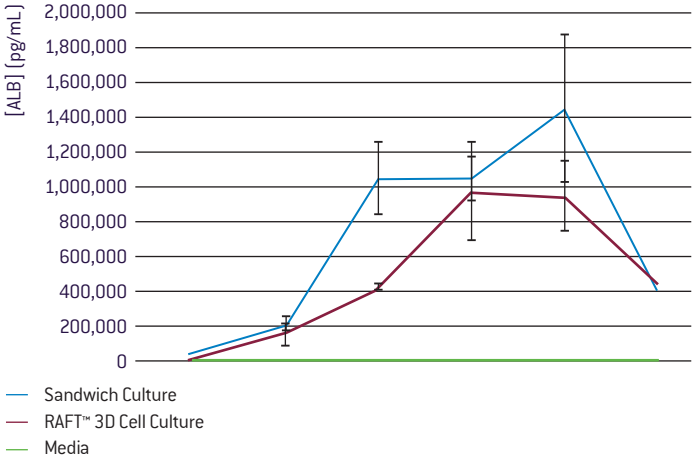


Figure 5

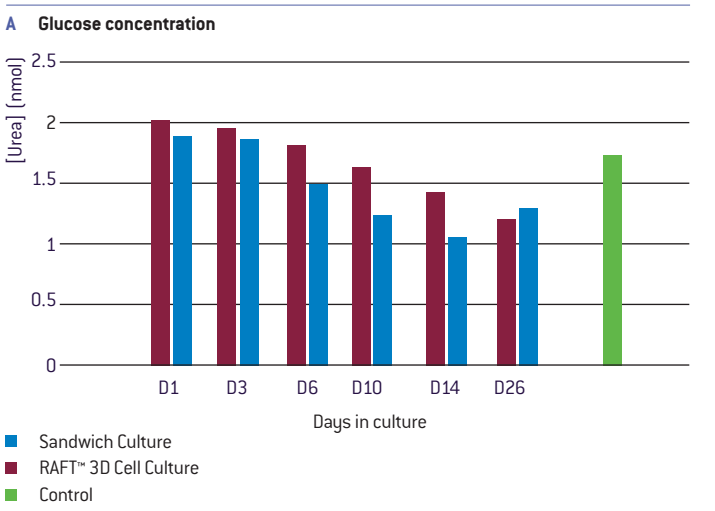
Albumin secretion by human hepatocytes in RAFT™ 3D Cell Culture and in Sandwich Culture are similar. Cells were plated at 45,000–65,000 cells per well in each respective model. Culture media was changed daily. Supernatants of 24-hour conditioned medium were collected on the culture days indicated. Culture medium from wells containing no cells used as a control.

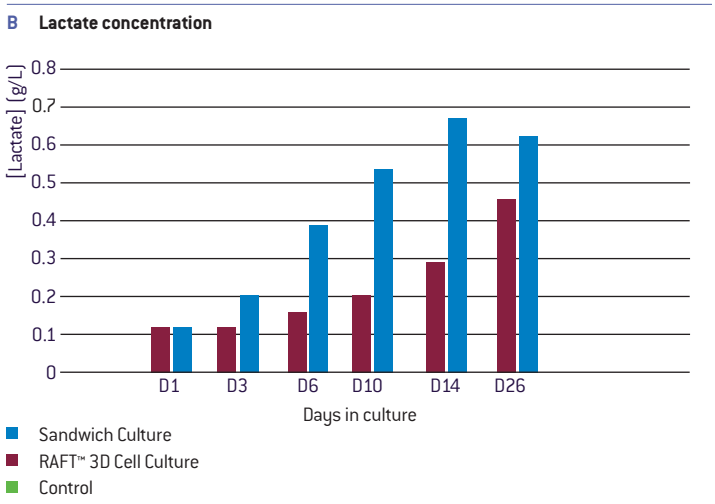
Glucose utilization and lactate and urea production by primary human hepatocytes is lower in RAFT™ 3D Cell Culture than in Sandwich Culture

Since primary human hepatocytes are differentiated cells and do not proliferate, an increased cellular uptake of glucose occurs with the most energy-requiring metabolic processes other than cell growth. In this case, hepatocytes in RAFT™ 3D Cell Culture require less glucose than those in Sandwich Culture, indicating a healthier physiological state of hepatocytes in RAFT™ 3D Cell Culture than in Sandwich Culture: although hepatocytes are more metabolically active in RAFT™ 3D Cell Culture than in Sandwich Culture (Figure 2B), their glucose consumption is lower (Figure 6A).

Lactate is the product of anaerobic glycolysis and is correlated with glucose consumption.[12] The greater the glucose consumption, the greater the lactate production (Figure 6B).

Basal urea production measured over 26 days showed increase in both RAFT™ 3D Cell Culture and Sandwich Culture up to day 14, but decreased in Sandwich Culture and maintained in RAFT™ 3D Cell Culture when measured on day 26 (Figure 6C).





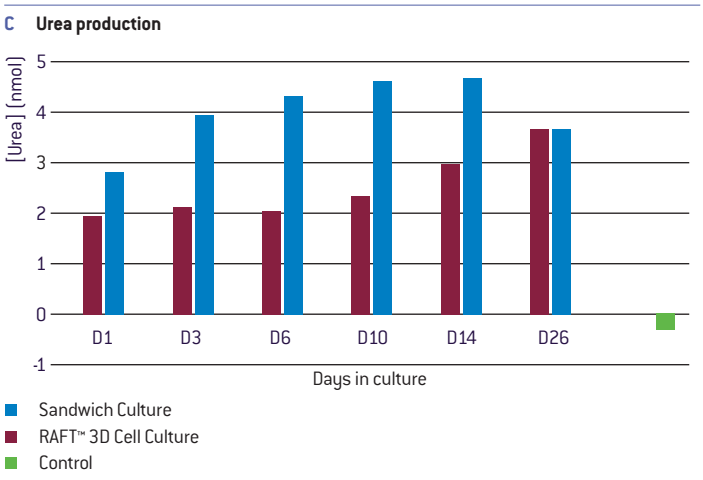


Figure 6
Glucose utilization and lactate and urea production by primary human hepatocytes is lower in RAFT™ 3D Cell Culture than in Sandwich Culture. Cells were plated at 45,000–65,000 cells per well in each respective model. Cultures were refed daily. Supernatants of 24-hour conditioned medium were collected on the culture days indicated. Culture medium from wells containing no cells was used as a control. (A) Glucose concentration (B) lactate concentration (C) urea production.

Basal and induced (CYP3A4) CYP activities in human hepatocytes are higher for longer duration in RAFT™ 3D Cell Culture than in Sandwich Culture

The presence of functional Cytochrome P450 (CYP) enzymes is another important characteristic of healthy hepatocytes. These enzymes are found primarily in liver and intestinal cells. The CYPs belonging to the CYP 1, 2 and 3 families account for the metabolism of 70–80% of the clinically used drugs.[13] Of these enzymes, CYP3A4 metabolizes the majority of known drugs, CYP2B6 metabolizes approximately 10% of drugs and CYP1A2 metabolizes a growing number of newly developed drugs.[14, 15] Table 1 summarizes the inducer (CYP3A4) substrates and metabolites for each CYP isoform.

Basal and induced (CYP3A4) CYP activities in primary human hepatocytes were measured over 17 days. Figure 7 shows that using both indirect (Figures 7 A, 7 C and 7 E) and direct (Figures 7 B, 7 D and 7 F) activity measurements, the basal CYP activity is higher and more stable when hepatocytes are cultured in RAFT™ 3D Cell Culture. Figure 8 shows that, in addition to facilitating higher basal CYP activities, RAFT™ 3D Cell Culture allows for rifampicin induction levels of CYP 3A4 to be higher over a longer duration than in Sandwich Culture. This data is summarized in Table 2.

CYP450 Isoform	Inducer	Substrate	Metabolite
CYP1A2	-	100 µM phenacetin	Acetaminophen
CYP2B6	-	250 µM bupropion	OH-bupropion
CYP3A4	10 µM rifampicin	200 µM testosterone	6ß-hydroxytesterone

Table 1
CYP inducers, substrates and metabolites.

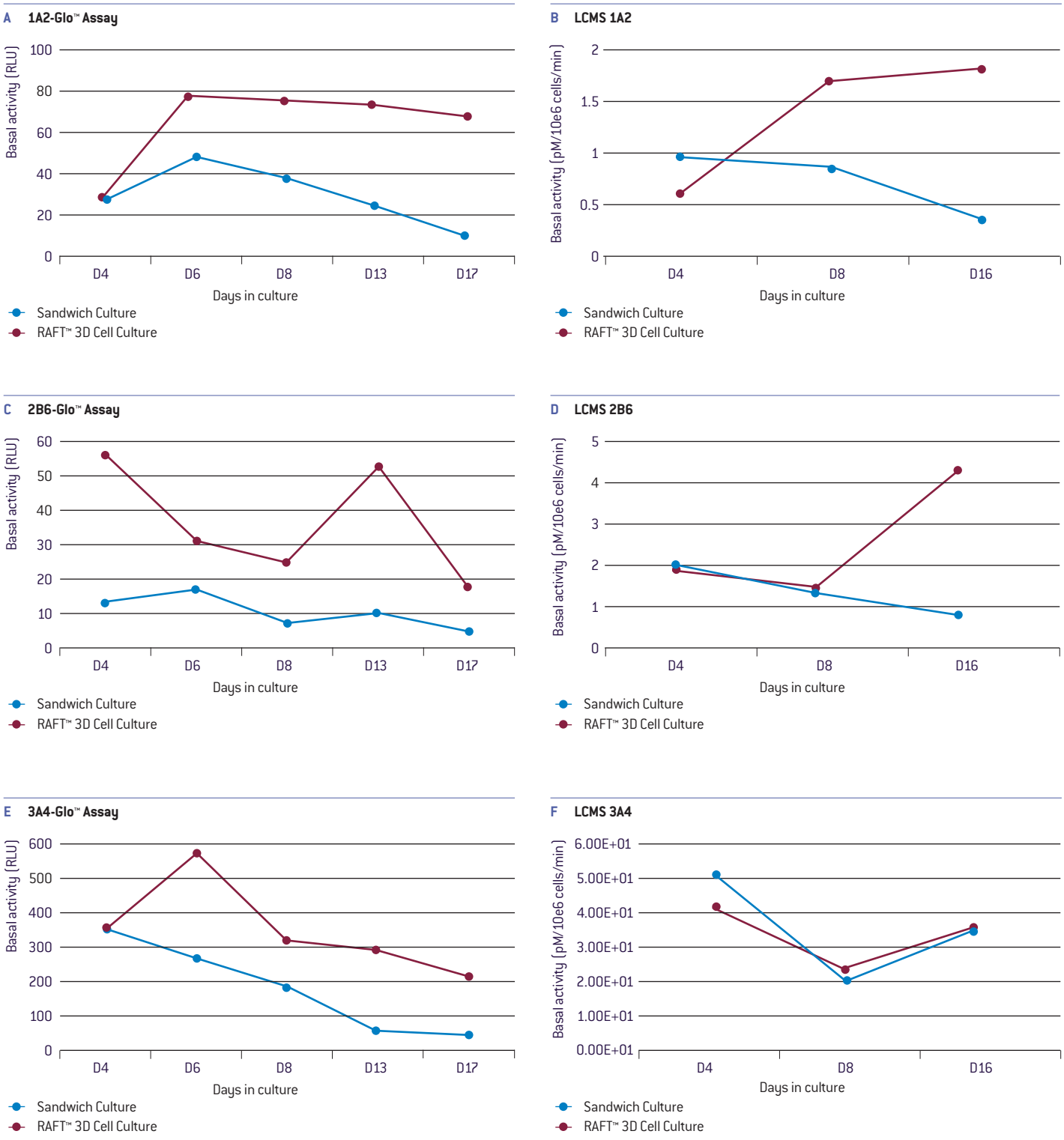
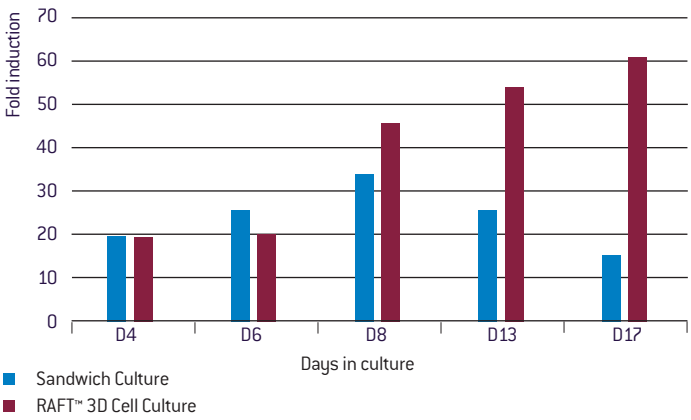


Figure 7

CYP450 Culture of human hepatocytes is higher for longer culture durations in RAFT™ 3D Cell Culture than in Sandwich Culture. Human hepatocytes were plated at 45,000–65,000 cells per well in RAFT™ 3D Cell Culture and in Sandwich Culture. Basal CYP activities were determined by analyzing supernatants from uninduced cells using the P450-Glo™ Assays (Figures 7A, 7C and 7E). In separate experiments the basal CYP450 activities were determined by incubating uninduced cells with the appropriate substrate (Table 1) and quantitating the metabolites by LCMS (Figures 7B, 7D and 7F).

A 3A4-Glo™ Assay



B LCMS 3A4

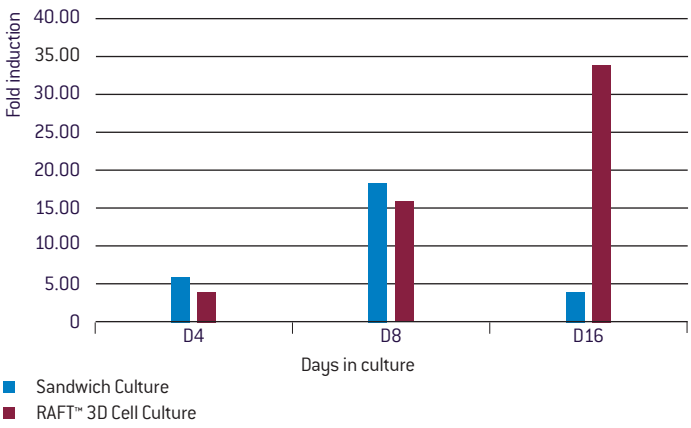


Figure 8
CYP450 activity of human hepatocytes is higher for longer culture durations in RAFT™ 3D Cell Culture than in Sandwich Culture. Human hepatocytes were plated at 45,000–65,000 cells per well in RAFT™ 3D Cell Culture and Sandwich Culture and incubated with the inducer rifampicin (Table 1) for 72 hours. Supernatants from induced and uninduced cells were analyzed using the P450-Glo™ Assays (**Figure 8**A). In a separate experiment, the induced and uninduced cells were incubated with the substrate testosterone (Table 1) and LCMS was performed to quantitate the metabolite 6ß-hydroxytestosterone (**Figure 8**B).

	P450-Glo™ Assay		LCMS	
	Sandwich Culture	**RAFT™3D Cell Culture**	**Sandwich Culture**	**RAFT™ 3D Cell Culture**
CYP3A4	15.3	61.8*	4.2	33.6

Table 2
Fold induction of CYP activity on day 16/17. Fold induction of CYP3A4 was calculated from the induction and basal CYP activity for both direct and indirect methods shown in **Figure 8**.
*Basal CYP levels higher in RAFT™ 3D Cell Culture

Conclusions

In this White Paper, we investigated the viability and performance of primary human hepatocytes in 3D vs. 2D cell culture systems beyond 7 days. We demonstrate that the RAFT™ 3D Cell Culture System is a robust and easy-to-use model for the culture of primary human hepatocytes. The collagen-based matrix of RAFT™ 3D Cell Culture provides a more *in vivo*-like environment than traditional 2D culture systems and better allows for the long-term maintenance of hepatocyte-specific functions. The primary human hepatocytes in RAFT™ 3D Cell Culture exhibited functions such as albumin and basal urea production similar to the 2D cell culture. For these studies, comparable viability data were obtained for several donors ranging in age from 2 years to 53 years.
Viability determinations and CYP analysis by indirect (P450-Glo™ Kits) and direct (LCMS) activity measurements show that hepatocyte metabolism is stabilized in RAFT™ 3D Cell Culture, enabling long-term toxicity analysis using primary hepatocytes. In addition, the RAFT™ 3D Cell Culture System allows for the construction of more complex liver models with the possible inclusion of non-parenchymal cells. The RAFT™ 3D Cell Culture System holds strong potential for drug testing and in predictive toxicity studies.

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