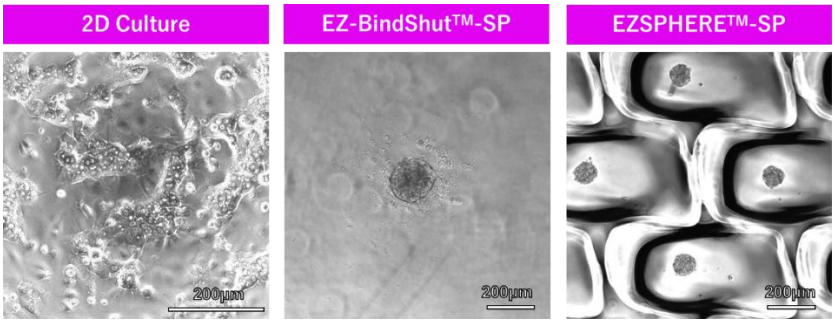
3D hepatocyte culture and hepatotoxicity screening in EZ-BindShut™ SP and EZSPHERE™ SP

Application Note

Introduction

HepaRG hepatocyte 3D (three-dimensional) culture are considered an effective tool for drug test model (Takahashi *et.al*., 2015 and Ramaiahgari *et.al*., 2017) . AGC has identified 3D culture protocol using common round bottom spheroid plates EZ-BindShut™-SP also micro-fabricated vessels EZSPHERE™-SP. 96-well format EZSPHERE has approximately 90 micro-wells (diameter = 500 μm, surface is coated with AGC original low cell adhesion coating). Therefor large-number of HeapRG 3D spheroids are created and cultured on EZSPHERE. By using EZSPHERE hepatotoxicity drug test can be performed with large-number of 3D HeapRG spheroids. HepaRG 3D spheroids culture on EZ-BindShut and EZSPHERE indicated in vivo hepatocyte structure and function. Additionally, hepatotoxicity drug test shown the different response between 2D and 3D culture.



AGC Techno glass products used in this application note.

Name	Format	Bottom shape	Culture surface	Cat. No.
EZ-BindShut SP	96-well microplate	round	Low cell adhesion SP coating	4870-800SP
EZSPHERE	96-well microplate	micro-fabricated	Low cell adhesion SP coating	4860-900SP
Cell culture plate	96-well microplate	Flat	Tissue culture treated	3860-096

AGC テクノグラス株式会社
コンシューマ本部 サイテック
https://iwaki.atgc.co.jp

Application 1: HepaRG 3D hepatic spheroid formation

1. Prepare cells and culture medium: cryopreserved differentiated HepaRG cells (BIOPREDIC International, #HPR116), plating medium (BIOPREDIC International, #ADD670) and maintenance medium (BIOPREDIC International, #ADD620) were obtained from KAC Co., Ltd.
2. Prepare the culture plate: For 3D hepatic spheroid culture, EZ-BindShut SP round bottom 96-well microplates and EZSPHERE 96-well microplates were used. For 2D culture, flat bottom TC-treated 96-well microplates were coated with collagen I (Nippi, #PSC-1-100-20).
3. HepaRG cells were thawed according to BIOPREDIC description.
4. After counting HepaRG cells, 1,500 cells/well were plated in EZ-BindShut SP 96-well microplates. 20,000 cells/well were plated in EZSPHERE SP 96-well microplates.
   Note: In EZSPHERE SP 96-well microplates, approximately 90 micro-wells designed for spheroids formation are micro-fabricated. On the calculation, 220 cells fall into each micro-fabricated well by the gravity and form spheroid in EZSPHERE.
5. The inoculated cells were carefully plated in a 37℃, 5%CO_2 incubator and allowed to incubate for 7 days.
6. On day4 and day6, 30 µl of 50 µl the plating medium was replaced by the maintenance medium in EZ-Bindshut SP. To avoid floating and loss spheroids, medium was no replaced in EZSPHERE for 7 days.

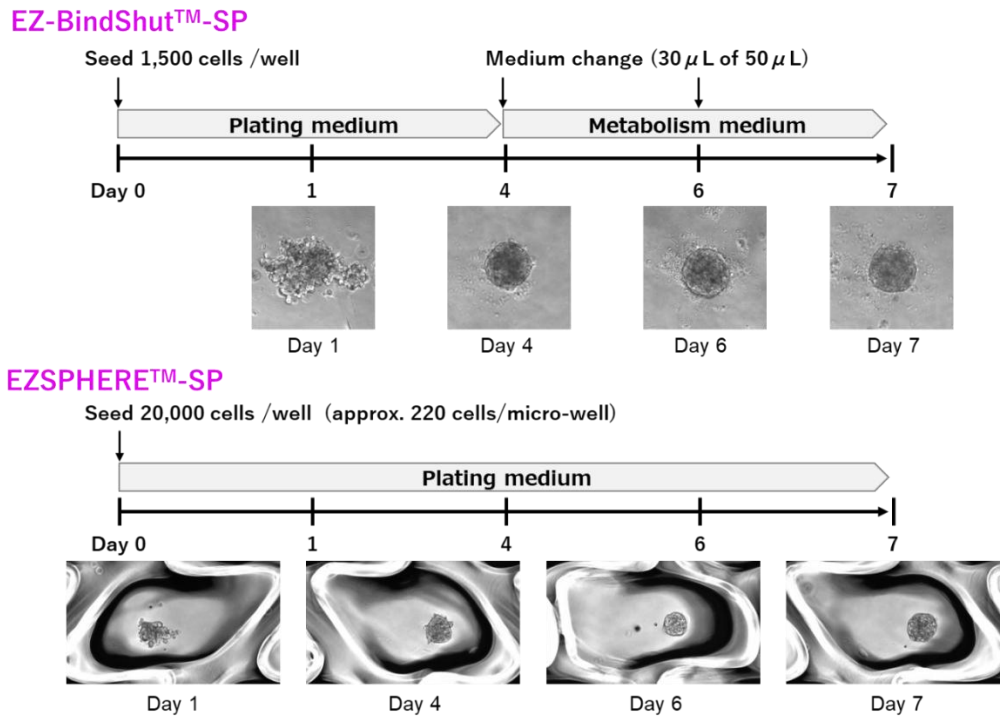


Figure 1. HepaRG 3D hepatic spheroids formation using EZ-BindShut and EZSPHERE

AGC テクノグラス株式会社
コンシューマ本部 サイテック
https://iwaki.atgc.co.jp

Application 2: Analysis of a bile canalicular in 3D hepatic spheroids

A few days after thaw and culture, HepaRG cells will form a coculture of hepatocyte and biliary epithelial-like cells (Gripon *et al.,* 2002). *In vivo* Hepatocyte and biliary cells are known to get typical polarity and form bile canalicular associated physiological functions. To identify bile canalicular is existing in HepaRG 3D spheroids, we stained multidrug resistance protein 2 (MRP2), a bile canalicular efflux transporter involved in drug and bile salt/acid transport (Kullak-Ublick *et al.*, 2002), which is involved in drug and bile salt/acid transport.

1. On day 8, HepaRG spheroids were harvested from EZ-BindShut SP and fixed with 4% paraformaldehyde for 10min.
2. Spheroids were washed with DPBS 3 times
3. Spheroids were permeabilized with 0.2% Triton-X in DPBS for 10 min followed by blocking with 1% BSA in PBST (DPBS with 0.2% Triton-X) for 30 min.
4. Spheroids were stained with anti-MRP2 antibody (1:250 dilution in 1%BSA-PBST, Abcam, #ab3373) overnight at 4℃.
5. Spheroids were washed with DPBS 3 times and stained with secondary antibody Goat anti-Mouse IgG Alexa 488 (1 µg/mL in 1%BSA-PBST, ThermoFisher, #A-11001) for 1 hour.
6. Spheroids were washed with DPBS 3 times followed by counterstain with Hoechst 33358 (Thermo, #H3570).
7. Spheroids were placed on EZVIEW glass bottomed plate and fluorescent immunostaining images were obtained using Fluorescent microscope Olympus IX 73.

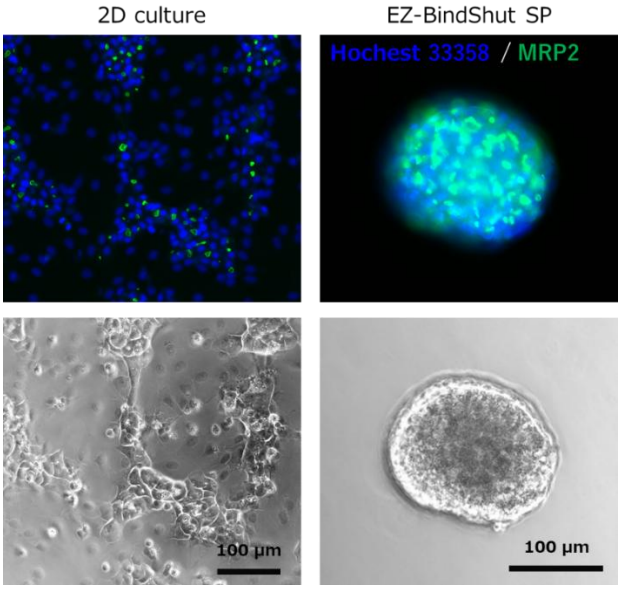


Figure 2. Microscopic images of 2D culture and 3D spheroid HepaRG.
Immunofluorescent staining of MRP2 (green) and nuclei (blue) above. Phase contrast image below. Scale bar indicates 100 µm.

AGC テクノグラス株式会社
コンシューマ本部 サイテック
https://iwaki.atgc.co.jp

Application 3: HepaRG spheroids in EZ-BindShut SP exhibit CYP3A4 induction

CYP3A4 is known as one of the most pharmacologically important cytochrome P450 enzymes involved in human liver metabolism. We observed CYP3A4 expression was induced by rifampin treatment to HepaRG 3D spheroids in EZ-BindShut-SP.

1. According to “Application 1”, HepaRG spheroids were formed from 1500 cells in each well of EZ-BindShut SP and cultured for 11 days.
2. During day 11 to day14, HepaRG spheroids were treated with variable level of rifampin to induce CYP3A4 mRNA expression.
   Note: Half volume of medium was changed with fresh medium containing variable level of rifampin every day.
3. On day 14, 6 to 8 spheroids were collected in a tube and conserved in -80℃ refrigerator.
4. cDNA was directly synthesized from spheroids using SuperScript IV CellsDirect cDNA Synthesis Kit (Invirogen #11750150).
5. Real time PCR was performed using TaqMan probes and TaqMan Universal PCR master mix (ThermoFisher) on Stratagene Mx 3000p (Agilent Technologies).

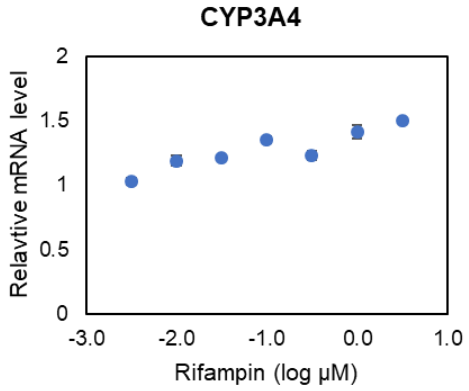


Figure 3. CYP3A4 induction assay

Application 4: Drug-induced hepatotoxicity

There is known of Chlorpromazine-induced hepatotoxicity in humans Chlorpromazine is extensively metabolized in the liver and kidneys. It is extensively metabolized by cytochrome P450 isozymes CYP2D6 (major pathway). Levels of drug-induced cytotoxicity was different in 3D HepaRG spheroid in and 3D HepaRG spheroids.

AGC テクノグラス株式会社
コンシューマ本部 サイテック
https://iwaki.atgc.co.jp

1. According to “Application 1”, HepaRG wells were seeded into 96-well microplates at 1500 cells/well (EZ-BindShut SP), 20000 cells/well (EZSPHERE SP) or 72000 cells/well (2D) and subsequently cultured for 7 days.
2. To treat 3D or 2DHepaRG with variable level of liver toxic drug, 50 times higher concentration (for each level) chlorpromazine was gently added each well containing HepaRG cells.
3. After 24 hours, cell viability of 3D HepaRG spheroids or 2D HepaRG was measured using the protocol for the Promega CellTiter-Glo® 3D Cell Viability Assay.

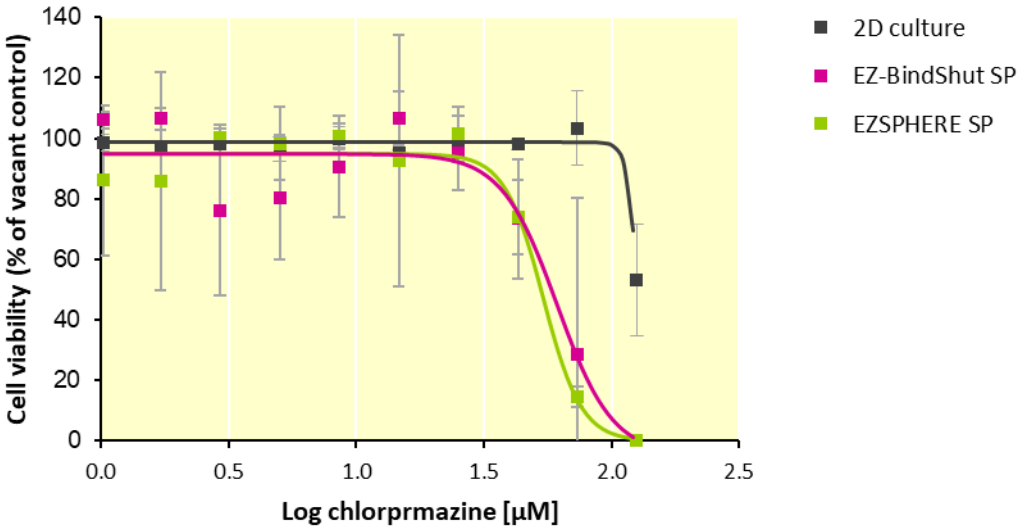


Figure 4. Hepatotoxicity drug test using 3D hepatocyte spheroids.

Reference

1. Gripon P, *et.al.*, (2002) **Infection of a human hepatoma cell line by hepatitis B virus.** Proc. Natl. Acad. Sci. U.S.A 99 (24) 15655-15660.
2. Takahashi Y, *et.al.*, (2015) **3D spheroid cultures improve the metabolic gene expression profiles of HepaRG cells.** Biosci Rep. 35(3):e00208.
3. Ramaiahgari SC, *et.al.,* (2017) **Three-Dimensional (3D) HepaRG Spheroid Model With Physiologically Relevant Xenobiotic Metabolism Competence and Hepatocyte Functionality for Liver Toxicity Screening.** Toxicol Sci. 160(1):189-190.

AGC テクノグラス株式会社
コンシューマ本部 サイテック
https://iwaki.atgc.co.jp