GrowDex®

Application Note **44**

UPMBIOFORE BEYOND FOSSILS

# Multiplex Analysis of 3D Liver Cell Cultures in GrowDex®

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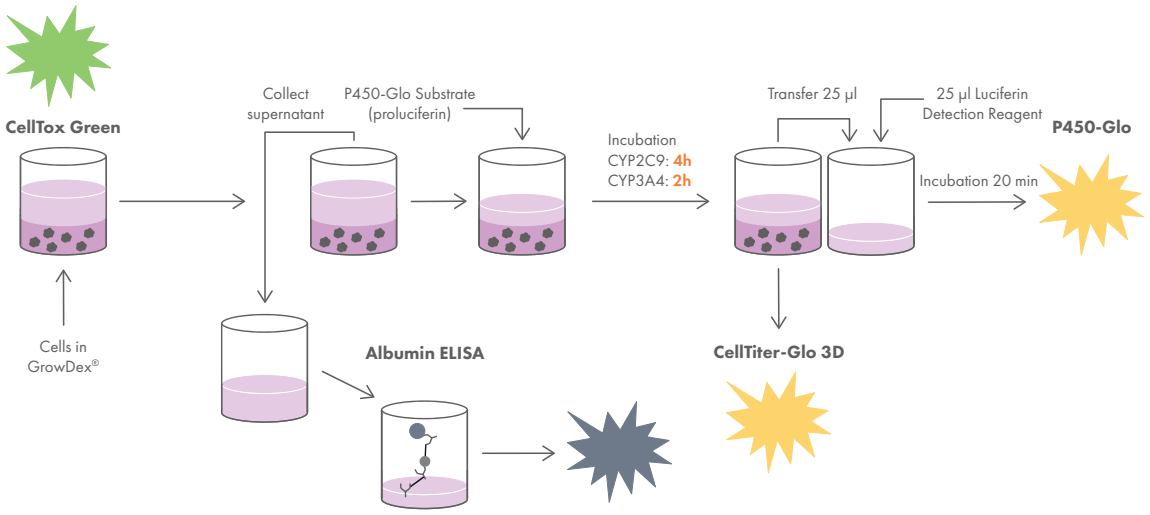
## INTRODUCTION

The liver is the main organ for metabolism of foreign substances, and drug-induced liver toxicity is a major reason for drug withdrawal from the market [1]. Traditional 2D models of primary human hepatocytes lose their viability and functionality within 1-2 weeks and do therefore not allow long-term drug exposure studies [2]. Therefore, novel human hepatocyte culture models that allow long term studies are needed for drug toxicity testing. These methods should be amenable to high-throughput and automation to allow testing of a large number of novel compounds at the same time. To save time and material and reduce the costs of drug development, it is a major advantage if these methods allow multiplexing, i.e. combining several readouts from the same sample or cell culture well.

GrowDex is an automation-friendly hydrogel for 3D cell culture, and hepatocytes cultured in GrowDex retain their functionality for several weeks. Promega's cell-based assays are user-friendly, straightforward protocols and can be combined to analyze different characteristics of the same culture at once. The aim of this study was to develop a culture protocol for HepaRG liver cells grown in GrowDex and a multiplexing protocol to quantify cell death and viability as well as hepatocyte functionality by testing cytochrome P450 activity and albumin secretion over the course of four weeks.

### Multiplex assay




Promega

UPMBIOMEDICALS

**MATERIALS**

- Differentiated HepaRG (Cat No. HPR116080, Biopredic)
- William's E medium (Cat No. 12551032, Gibco)
- GlutaMax (Cat No. 35050038, Gibco)
- HepaRG Thawing/Plating/General Purpose Medium Supplement with antibiotics (Cat No. ADD670C, Biopredic)
- HepaRG Maintenance/Metabolism Medium Supplement with antibiotics (Cat No. ADD620C, Biopredic)
- PhenoPlate 96-well, black, optically clear flat-bottom, ULA-coated (Cat No. 6055802, Revvity)
- 96 Well White/Clear Bottom Plate (Cat No. 10067581, Fisher Scientific).
- Low-retention pipet tips 1000 µl (Cat No. 11535454, Fisher Scientific)
- Low-retention pipet tips 200 µl (Cat No. 70.3031.275, Sarstedt)
- GrowDex®, 1.5% (Cat No. 100 103 005, UPM Biomedicals)
- CellTox™ Green Cytotoxicity Assay (Cat No. G8742, Promega)
- P450-Glo CYP3A4 Assay with Luciferin-IPA (Cat No. V9002, Promega)
- P450-Glo CYP2C9 Assay with Luciferin-H (Cat No. V8792, Promega)
- CellTiter-Glo® 3D Cell Viability Assay (Cat No. G9682, Promega)
- Human Albumin ELISA Kit (Cat No. ab179887, abcam)
- Rifampin (Cat No. 15473539, Fisher Scientific)
- Itraconazole (Cat No. S2476, Selleckchem)
- ATP solution 100 mM (Cat No. 10304340, Thermo Fisher)
- Victor Nivo multimode plate reader (Cat No. HH35000500, Revvity)
- CertusFlex (Fritz Gyger AG)
- GraphPad Prism 10.0.3 (GraphPad Software)

**METHODS**

1. A 1% solution of GrowDex was prepared using the HepaRG Thawing/Plating/General Purpose Medium. Briefly, to each ml media in a reaction tube 2 ml GrowDex were added, then mixed thoroughly by stirring and pipetting whilst avoiding bubble formation.
2. Differentiated HepaRG cells were thawed according to the manufacturer's instructions, counted, and mixed with the 1% GrowDex solution and with media to reach 750,000 cells/ml in a 0.5% GrowDex solution. This solution was transferred to black ultra low adhesion 96-well plates at 100 µl/well, and 100 µl HepaRG Thawing/Plating/General Purpose medium were gently added on top. One plate per analysis timepoint was prepared.
3. To prepare samples for blank/background measurements, six wells on each plate were filled with 100 µl cell-free 0.5% GrowDex in HepaRG Thawing/Plating/General Purpose Medium, and 100 µl medium were added on top.
4. Following a 2 d incubation, medium was changed to HepaRG Maintenance/Metabolism medium containing 1:500 CellTox-Green dye. Due to the GrowDex in the wells, the final concentration was 1:1000. This and all following media changes were also performed for the wells containing GrowDex without cells.
5. HepaRG Maintenance/Metabolism medium was renewed three times a week, then containing 1:1000 CellTox-Green dye.
6. Before each change of medium, cell death was quantified by recording the fluorescence of the CellTox-Green dye in a plate reader with settings according to the kit instructions. The cell-free GrowDex-only wells were used for background measurement.
7. After 7/14/21/28 d spheroid culture, the cells in one plate were treated with Rifampin or Itraconazole, or control-treated with media, all of these containing 1:1000 DMSO. The drug concentration in the supernatant was doubled compared to the final concentration, taking the 1:2 dilution by the 100 µl GrowDex into account.
8. After 2 d drug treatment, cultures were analyzed for cell death as described above. Then, the supernatants were collected for further analysis (e.g. quantification of albumin secretion) and stored at -20 °C, and CYP activity as well as cell viability were analyzed as described below.
9. CYP3A4 activity: The P450-Glo kit components were thawed and the Luciferin Detection Reagent was reconstituted according to the kit instructions. Then, HepaRG Maintenance/Metabolism medium containing the CYP3A4 substrate was prepared. To reach the final dilution 1:1000 (3 µM) on the cells, the concentration in the media was tripled, meaning a dilution of 1:333.33. 50 µl of this solution were gently added on top of the cultures in GrowDex, as well as into the wells containing GrowDex only. The plates were incubated at 37 °C/5% CO2 for 2 h. Following this incubation, 25 µl of the supernatant were transferred to a new, white walled 96-well reaction plate, and 25 µl of the reconstituted Luciferin Detection Reagent were added with Certus Flex. The plate was incubated at room temperature for 20 min, and luminescence was recorded in a plate reader. The results were blank-corrected and normalized to the ATP content of the wells determined by CellTiter-Glo 3D.

10. Cell viability: The CellTiter-Glo 3D reagent was thawed at 4 °C overnight, equilibrated at room temperature, and mixed by gently inverting before use.
    A dilution series of ATP standard in HepaRG Maintenance/Metabolism medium was prepared at concentrations 0.5 nM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1 µM, 5 µM, 10 µM, 50 µM, 100 µM. 100 µl of these were added in duplicates to empty wells on the 96-well plate. The plate was equilibrated to room temperature, the leftover supernatant was removed from the cells/GrowDex, and 100 µl CellTiter-Glo 3D reagent were added to each well. After mixing for 5 min at room temperature the incubation was continued for 25 min, and the luminescence recorded according to the kit instructions. NB: extended shaking can improve the signal intensity. The ATP content of the samples was extrapolated from the ATP standard curve.
11. Quantification of albumin secretion: The albumin content of cell culture supernatants was determined via ELISA. The supernatants were thawed, centrifuged at 2000xg for 10 min and diluted 1:200. The assay was performed according to the kit instructions. The albumin content was extrapolated from the standards, and the results were normalized to the ATP content of the wells.

## RESULTS

HepaRG cells in GrowDex started clustering within 1-2 days after plating. After 5-7 d of culture, spheroids of 50-70 µm had formed **(Fig. 1)**, and this size remained stable for the duration of the experiment. After 1/2/3/4 weeks, the cells were treated with a standard CYP inhibitor (Itraconazole) and inducer (Rifampin), and readouts were performed after 2 d incubation with the compounds.

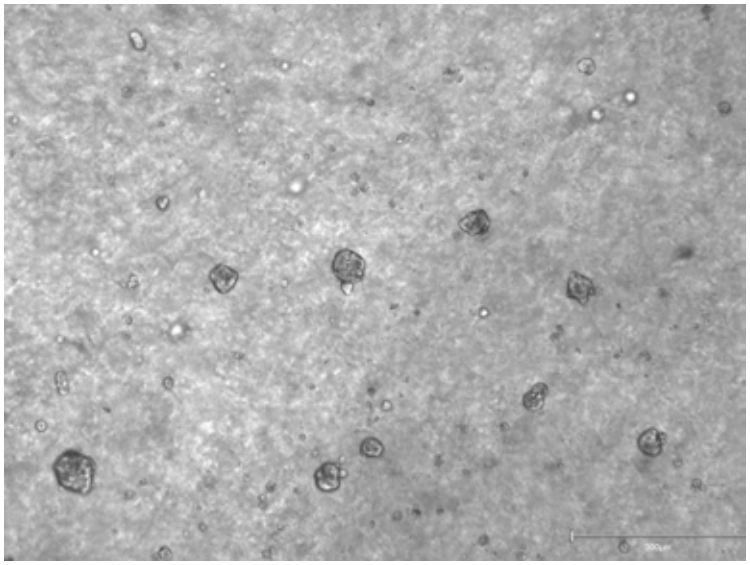


**Figure 1.** HepaRG spheroids in 0.5% GrowDex 9 days after plating. Scale bar 300 µm.

**Setup of multiplexing assay.** Adding the CellTox-Green reagent to culture media allowed for an easy quantification of cell death at any time during the experiment without further pipetting. Therefore, this was the first assay to be performed in the multiplexing protocol, followed by P450-Glo and finally by CellTiter-Glo 3D. Supernatants of the cultures were collected after CellTox-Green measurements and before quantification of CYP activity and can be used for a multitude of different readouts, as exemplified here with quantification of albumin secretion. The P450-Glo assay was performed according to the nonlytic protocol, however, the incubation time with proluciferin had to be extended from 1 h to 2 h to obtain stronger signals that allowed more sensitive differentiation between treatments. Performing the nonlytic protocol of P450-Glo allowed for determination of cell viability via CellTiter-Glo 3D, the results of which were then used for normalization of CYP activity and albumin secretion.

**Cytotoxicity of compounds.** While Itraconazole treatment after 3 weeks culture time seemed to have some cytotoxic effect, the treatments generally did not induce cell death **(Fig. 2A)**, therefore allowing detailed analysis of hepatocyte-specific activity.

This was verified by the cell viability assay CellTiter-Glo 3D. As expected for a non-dividing cell line, cell viability decreased over time, however, it was not affected by the treatments **(Fig. 2B)**.

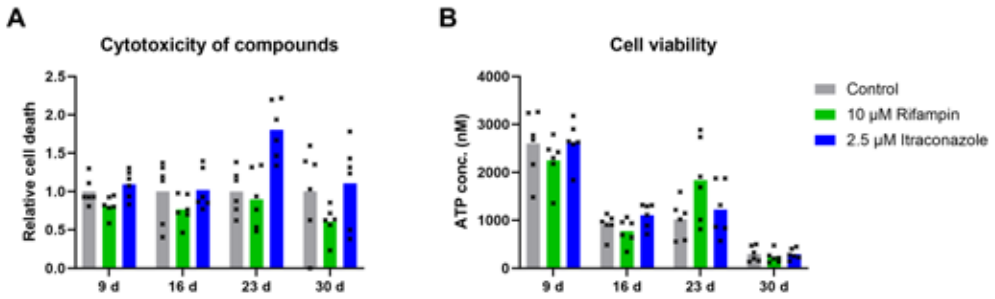



**Figure 2.** Effect of compound treatment and of long-term culture on HepaRG spheroids. Cells were cultured in GrowDex for the indicated times and compound-treated for 2 d before cytotoxicity and viability were quantified. Cyotoxicity relative to control (A), and cell viability as ATP concentration (B). N=6.

**CYP3A4 activity.** The basal CYP3A4 activity decreased over time as shown in **figure 3A**, however, it could be induced/inhibited throughout the 30 d experiment **(Fig. 3B)**. Treatment with 10 µM Rifampin induced the activity by factors 2.48, 1.82, 3.78, 3.17 after 1, 2, 3, 4 weeks culture time, respectively. Itraconazole treatment led to activities of 0.08, 0.14, 0.23, 1.06 relative to control at the same timepoints. Even though CYP3A4 activity was not reduced by Itraconazole treatment after 4 weeks in culture, this shows that modulation of CYP enzyme activity is feasible in long-term HepaRG cultures in GrowDex.

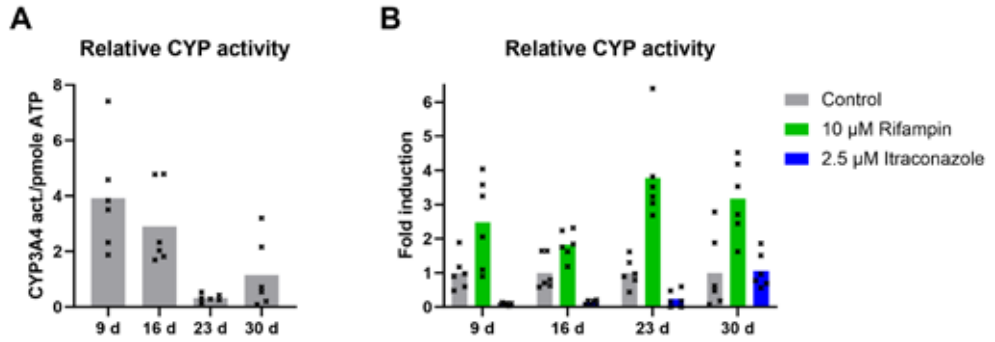



**Figure 3.** CYP3A4 activity. HepaRG cells were cultured in GrowDex for the indicated times and compound-treated for 2 d before CYP3A4 activity was quantified. Relative CYP3A4 activity of control-treated cells (A). Induction and inhibition of CYP3A4 activity shown as relative to control (B). N=6

**Albumin secretion.** Albumin secretion slightly increased at day 16 compared to day 9, followed by a decrease at the later time points **(Fig. 4)**. Treatment with CYP inducer and inhibitor did not affect the secretion of albumin.

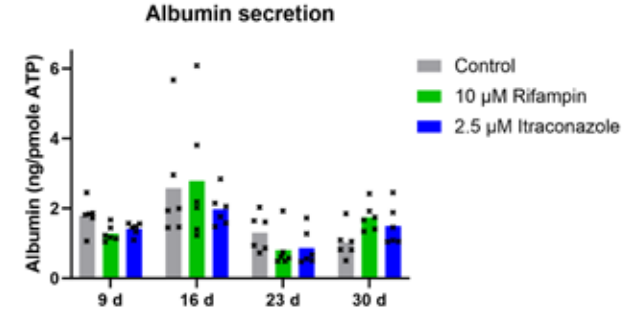



**Figure 4.** Albumin secretion. HepaRG cells were cultured in GrowDex for the indicated times and compound-treated for 2 d before supernatants were collected, stored at -20 °C, and analyzed for albumin content. Results were normalized to the ATP content of the cultures. N=6.

## CONCLUSIONS

This study shows that a 3D HepaRG hepatocyte model can be successfully maintained in GrowDex for up to 30 days. Consistent with expectations for a non-proliferative cell line, a decline in cell viability was observed over time. However, the liver cells continued to demonstrate specific functional capabilities, evidenced by the sustained activity of CYP3A4 and the secretion of albumin, although both parameters showed a reduction over time. Importantly, CYP3A4 activity can be modified by drug treatment throughout the 30 d incubation.

This model can be analyzed by multiplexing of assays to quantify cell death, CYP activity, as well as cell viability (employing CellTox-Green Cytotoxicity Assay, P450-Glo CYP3A4 Assay, and CellTiter-Glo 3D Cell Viability Assay, respectively), and additionally by supernatant-based readouts like ELISA to quantify albumin secretion. We demonstrated that these assays are fully compatible with GrowDex hydrogels, only a minor modification (extension of incubation time) of the standard protocol for P450-Glo was necessary. The capability to perform these assays simultaneously in a single experiment offers several benefits, such as generating more biologically relevant data, conserving precious samples, reducing experimental time, and minimising reagent use. It also streamlines workflows and cuts down on the time and costs associated with toxicity testing approaches. One step, the addition of the CYP detection reagent, was performed here with an automatic dispenser. The automation-friendly characteristics of GrowDex and of the assays can allow even more automation of the process, which would increase speed and reproducibility whilst reducing costs. We have also optimized the P450-Glo protocol for CYP2C9 in this model, showing the versatility of the multiplexing protocol. The liver cell model and multiplexing of assays described here can be used in a multitude of applications in the future. This includes liver toxicity studies, as well as quality control assays to evaluate batches of primary human hepatocytes.

In addition, more supernatant-based readouts can be easily added to this model without further optimization of the culture or multiplexing conditions. This would enable measurement of even more parameters depending on the scientific question.

## REFERENCES


1. *Allison, R., Guraka, A., Shawa, I.T., Gyan Tripathi, G., Moritz, W., Kermanizadeh, A.* **(2023)**. *"Drug induced liver injury – a 2023 update." J Toxicol Environ Health B Crit Rev 26(8) https://doi.org/10.1080/10937404.2023.2261848*
2. *Yang, S., Ooka, M., Margolis, R.J., Xia, M.* **(2023)**. *"Liver Three-Dimensional Cellular Models for High-Throughput Chemical Testing." Cell Reports Methods 3(3) https://doi.org/10.1016/j.crmeth.2023.100432*





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