CryoShield™ Cryopreserved HepG2 Monolayers: Testing Differentiated Hepatic Functions and Toxicological Challenges

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HepG2 cells perform many differentiated hepatic functions and, hence, are commonly used in hepatotoxicity studies and drug metabolism. CryoShield™ technology now allows the cryopreservation of HepG2 cells adhered to 24- and 96-well plates, minimising the time required to perform assays for drug discovery and assay development. The ASSAY READY cryopreserved HepG2 plates are simply removed from the ultra-low temperature freezer, thawed with cell culture medium, and can be used to test for differentiated hepatic function or drug toxicity within 24 hours. HepG2 cells retained normal levels of differentiated hepatic functions including urea cycle activity, Cytochrome P450 basal activity and lipid droplet formation 24 hours post-thaw. Toxicological challenges against 6 pharmacologically active substances shows near-identical IC_{50} values between non-frozen, conventionally cultured, HepG2 cells and CryoShield™ cryopreserved cells. ASSAY READY HepG2 plates are ready for deployment in drug discovery and provide major time savings by removing all culturing steps, along with improved inter-assay variability and reducing single use plastic wastage.

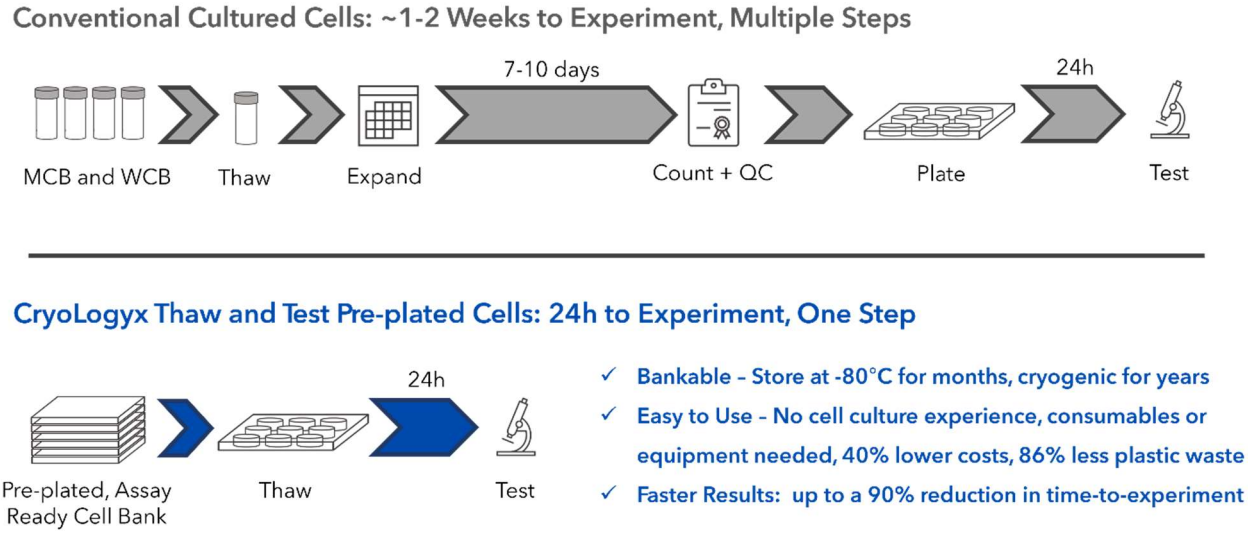

Figure 1. Time required from cryopreservation to testing/analysis. A comparison between suspension cryopreserved and CryoShieldTM cryopreserved HepG2 cell workflow.

Introduction

HepG2 cells perform many differentiated hepatic functions and, hence, are the most widely used cells in hepatotoxicity studies and drug metabolism. However, drug screening times are prolonged by routine cell culture and the time required to propagate cells from frozen suspension stocks to attain the desired adhered morphology. CryoShield™ allows the cryopreservation of HepG2 cells directly adhered to tissue culture plastic to remove these time-expensive barriers (**Figure 1**), whilst also minimising inter-assay variability due to genetic drift from passaging cells and single-use plastic waste. The ASSAY READY HepG2 plates are simply removed from the -80 °C freezer, thawed with cell culture medium and used in 24 hours.

Cryopreserved HepG2 monolayers, ready-to-use from the freezer, accelerate and simplify the discovery of pharmaceutically active compounds, biocompatibility testing, assay development, and discovery of cell signalling and disease pathways. In previous application notes, we demonstrated that CryoShield™ cryopreserved HepG2 monolayers retain normal cellular functions and viability and are compatible with multiple assays within 24 hours of thawing. This application note further expands on the utility of ASSAY READY HepG2 plates by illustrating that the cryopreserved HepG2 monolayers also possess normal levels of differentiated hepatic functions, which are key to studying drug metabolism. Toxicological challenges against 6 pharmaceutically

relevant drugs revealed that near-identical IC_{50} values can be obtained with CryoShield™ cryopreserved cells compared to conventionally cultured cells, highlighting that ASSAY READY HepG2 plates can be used to study drug-induced toxicity in a quarter of the timeframe.

Materials and Methods

HepG2 cells were plated in 96-well plates, at multiple cell densities, and cryopreserved with CryoShield™. The plates were stored in a -80 °C freezer until required and were thawed with Eagle's minimum essential medium supplemented with 10% FBS, 1% PSA and 1% NEAA. The plates were incubated in a humidified environment at 37 °C and 5% CO_2 for 24 hours before the following assays were completed.

Urea secretion into the cell culture medium was measured using a Urea Assay kit (Merck) at days 1, 4 and 7 post-thaw. Lipid droplet formation in the absence (base level) and presence of 0.5–1 mM free fatty acid solution (1:1 of sodium palmitate: sodium oleate), for 24 hours, was imaged after staining with Nile red using fluorescence microscopy. Fluorescence measurements were also recorded. CYP3A4 and CYP2C9 basal levels were assessed using

HepG2 cells (15k cells/well) by treating them with acetaminophen, diclofenac, doxorubicin, metformin, phenformin and valproic acid for 24 hours. Cell viability was quantified by a resazurin reduction assay to determine IC_{50} values. All experiments were completed with non-frozen, conventionally cultured HepG2 cells for comparison.

The urea cycle of CryoShield™ cryopreserved HepG2 is undisturbed

HepG2 cells derive from a hepatocellular carcinoma and perform many differentiated hepatic functions including albumin secretion and urea production. The urea cycle is an important detoxification pathway that forms urea from ammonia. Ureogenic capacity over-stimulation and inhibition can be influenced by pharmacologically active substances, *in vitro* environmental changes (substrates and co-cultures) and genetic modifications. Urea cycle activity of CryoShield™ cryopreserved HepG2 remained normal following thawing at days 1, 4 and 7, with urea levels in cell culture medium increasing with time, **Figure 2**.

CryoShield™ cryopreserved HepG2 cells can be used to study lipotoxicity and triglyceride accumulation

HepG2 cells can store triglycerides in lipid droplets present in the cytosol. An increase in lipid droplet formation due to free fatty acid solutions or drugs is a proposed marker for steatosis and lipotoxicity. CryoShield™ cryopreserved HepG2 cells respond normally to the presence of free fatty acids, accumulating them as triglycerides into lipid droplets, which were stained with Nile red, **Figure 3A**. Fluorescence measurements confirmed an increase in lipid droplet content, **Figure 3B**. At high free fatty acid concentrations lower cell densities were observed consistent with lipotoxicity onset. Thus, CryoShield™ cryopreserved HepG2 cells can be used as a steatosis model and to study mixtures and drugs that may induce lipotoxicity.

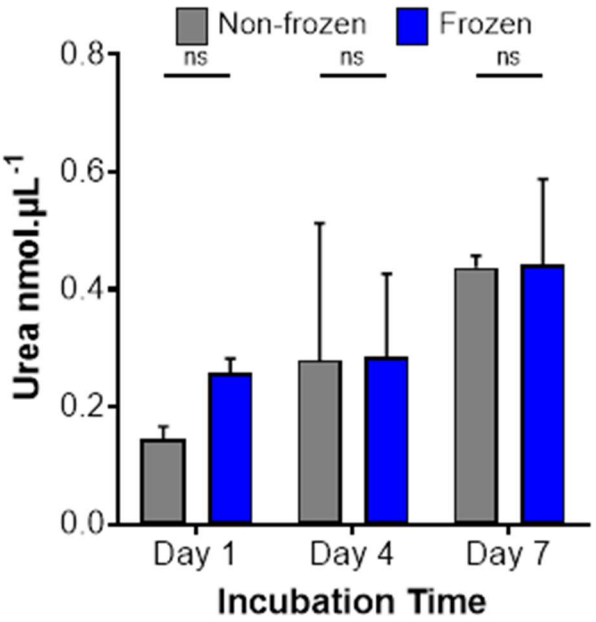

Figure 2. Urea secretion measurements. CryoShield™ cryopreserved HepG2 cells (30k cells per well) were thawed and urea measurements were recorded after days 1, 4 and 7. Urea measurements for non-frozen, conventionally cultured HepG2 cells were provided for comparison.

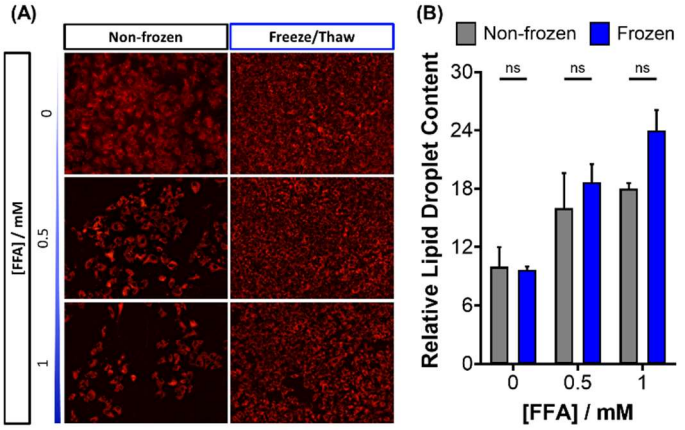

Figure 3. Triglyceride accumulation in lipid droplets from free fatty acid incubation. CryoShield™ cryopreserved HepG2 cells (30k cells per well) and conventionally cultured HepG2 cells were incubated with 0 (basal) to 1 mM of a free fatty acid solution and stained with Nile red. Cells were (**A**) imaged with fluorescence microscopy and (**B**) fluorescence measurements were recorded to determine relative lipid droplet content.

Cytochromes P450 basal levels remain normal following thawing

Cytochrome (CYP) enzymes are mainly responsible for phase I metabolism of xenobiotics in the liver. HepG2 cells present measurable quantities of CYPs involved in the metabolic activation and inactivation of drugs, with CYP3A4 being responsible for 50% of all drug metabolism. Thus, many drug screening applications rely on CYP measurements to study drug clearance and metabolites, drug-drug interactions and human hepatotoxin detection. Furthermore, they can be used for the rapid identification of CYP enzymes relevant to a specific reaction or screening for CYP enzyme inducers and inhibitors. CYP3A4 and CYP2C9 activity were measured to illustrate that ASSAY READY HepG2 plates possess normal CYP activity levels 24 hours post-thaw, **Figure 4**, and, thus, can be used for drug metabolism testing and to study drug-drug interactions.

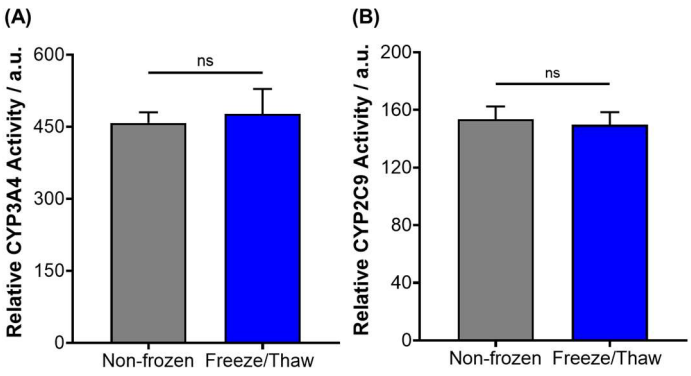

Figure 4. Basal CYP P450 activity. (**A**) CYP3A4 and (**B**) CYP2C9 basal activity levels were measured 48 hours following thawing of CryoShield™ cryopreserved HepG2 cells (30k cells per well). The CYP activity of non-frozen HepG2 cells were also completed for comparison.

As previously mentioned, HepG2 cells are commonly used in hepatotoxicity studies and drug metabolism. Thus, we tested HepG2 cells against a panel of different pharmacologically active compounds in a typical 24-hour drug toxicity screening experiment. Drug screening times are reduced by 4-fold by using ASSAY READY HepG2 plates that do not require routine cell culture. **Figure 5** illustrates the workflow of a typical 24-hour drug screening assay. The dose-response curves and IC_{50} values of all six pharmacologically active compounds were similar between ASSAY READY HepG2 plates and conventionally cultured cells, **Figure 6**, demonstrating that drug screening can be completed in a fraction of the time to determine the required drug dosage and frequency, as well as its therapeutic index.

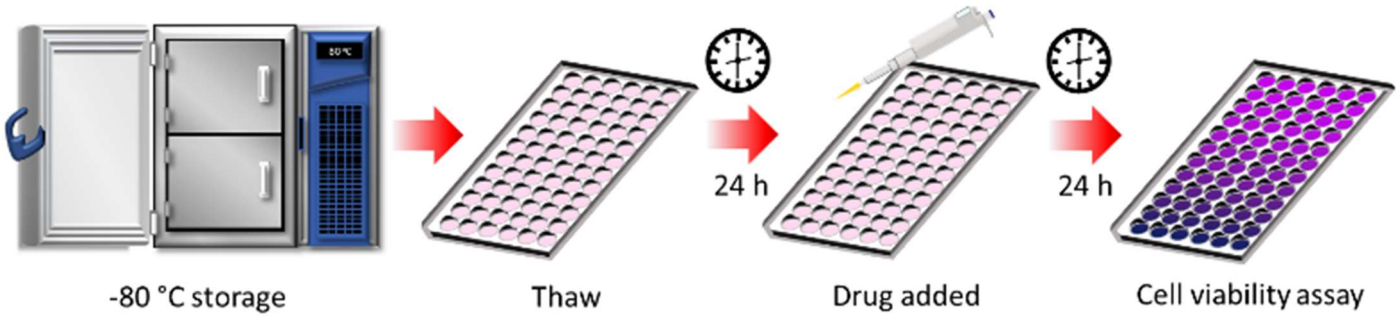

Figure 5. Workflow of a typical 24-hour drug screening assay. ASSAY READY HepG2 plates were removed from the -80 °C freezer, thawed and the drugs were applied 24 hours later. Following 24 hours of incubation with the test compounds, a resazurin reduction assay was completed to measure cell viability and determine IC_{50} values.

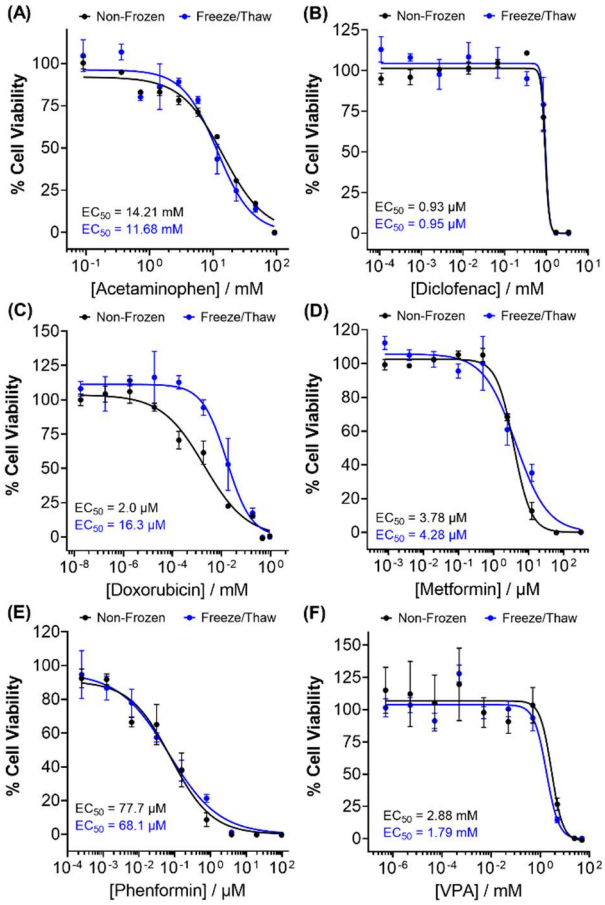

Figure 6. Dose-response curves for pharmacological active drugs. CryoShield™ cryopreserved HepG2 cells and conventionally cultured cells (15k cells per well) were treated with (**A**) acetaminophen, (**B**) diclofenac, (**C**) doxorubicin, (**D**) metformin, (**E**) phenformin and (**F**) valproic acid for 24 hours and cell viability was measure using a resazurin reduction assay. Dose-response curves were plotted to determine IC_{50} values.

Conclusions

ASSAY READY HepG2 plates, produced using CryoShield™, remove the unnecessary burden of routine cell culture by providing bankable cells, in a -80 °C freezer, which are directly adhered to tissue culture plastic and ready to use 24 hours following thawing. The CryoShield™ cryopreserved HepG2 cells present normal levels of differentiated hepatic functions including urea cycle activity, lipid droplet formation and cytochrome's P450 basal activity, crucial functions required for studying drug metabolism (drug clearance, metabolites, and interactions). Drug toxicity screening experiments revealed that cryopreserved HepG2 cells can produce almost identical dose-response and IC_{50} results compared to conventionally cultured cells but in a quarter of the timeframe. ASSAY READY HepG2 plates could revolutionise the field of drug discovery, offering the potential to maximise time spent on research-based activities, whilst minimising inter-assay variability caused by routine cell culture.