CryoShield™ Cryopreserved HepG2 Cell Monolayers Recovery of Viable and Functional Cells in 24 hours for Assay Development and Use

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Cell monolayers underpin the discovery and screening of new drugs and allow for fundamental studies of cell biology and disease. Current cryopreservation technologies do not allow cells to be stored attached to tissue culture plastic. Hence, cells must be thawed from suspension, cultured for several days or weeks, and finally transferred into multiwell plates for the desired application, requiring extensive time. In this study, we demonstrate that CryoShield™, Cryologyx's patented formulation, allows the cryopreservation of HepG2 cells adhered to 24- and 96- well plates to produce ASSAY READY HepG2 plates. After 24 hours of thawing, over 80% of HepG2 cells are recovered with healthy adherent morphology and normal proliferative capacity and metabolic function. Minimal well-to-well variation in cell density is observed to reduce the risk of assay variability. We demonstrate that ASSAY-READY HepG2 plates can be used in imaging-based, enzymatic leakage and cell viability assays crucial for drug toxicity studies. ASSAY-READY HepG2 plates, produced using CryoShield™, offers the potential to transform the field of drug discovery and assay development by removing unnecessary routine cell culture, reducing assay time and increasing assay flexibility.

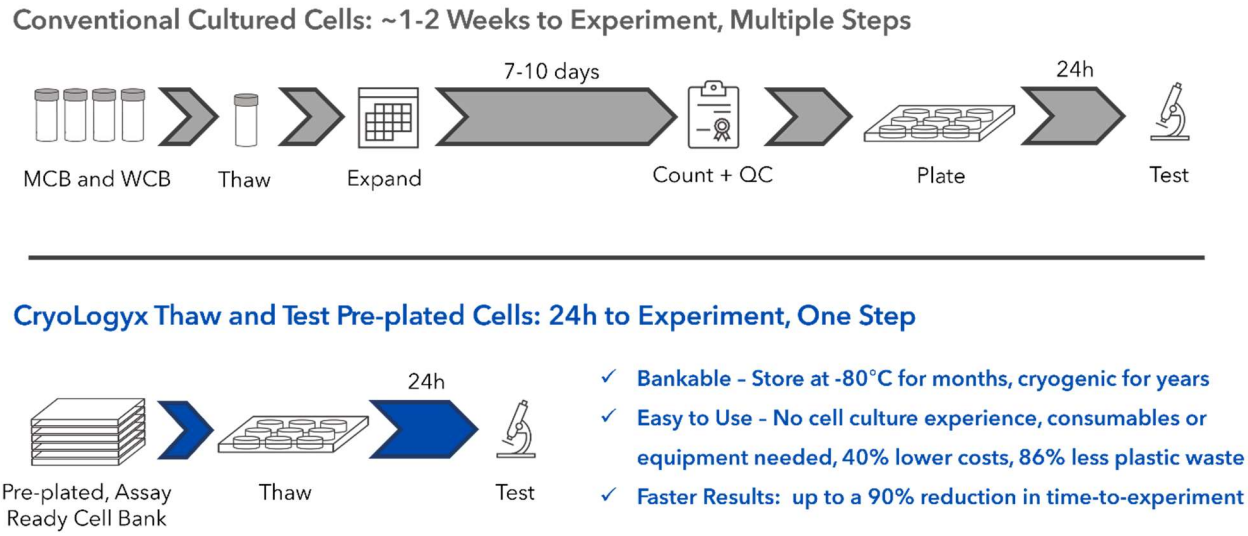

Figure 1. Time required from cryopreservation to testing/analysis. *A comparison between suspension cryopreserved and CryoShieldTM cryopreserved HepG2 cell workflow.*

Introduction

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. By cryopreserving cells in the storage format required by cell biologists, the time required to propagate cells from frozen stocks to attain the desired adhered morphology and cell density is removed, maximising time spent on 'value-adding' research-based activities (**Figure 1**). Inter-assay variability due to genetic drift from passaging cells and single-use plastic waste is also minimised. Simply remove the pre-plated cells from the freezer, thaw them with cell culture medium and they're ready to use in 24 hours.

Conventional cryoprotectants are unable to cryopreserve cells adhered to well plates. CryoShield™ overcomes the cold stress-induced factors that limit cell recovery and viability following the thawing of cells cryopreserved directly adhered to tissue culture plastic, producing a cell-based product that can be stored in a -80 °C freezer and requires minimal handling.

In this application note, "CryoShield™ Cryopreserved HepG2 Cell Monolayers: Recovery of Viable and Functional Cells in 24 hours for Assay Development and Use", we demonstrate that CryoShield™ cryopreserved HepG2 monolayers in 24- and 96-well plates, retain normal cellular functions and are compatible with multiple assays within 24 hours of thawing. ASSAY READY HepG2 plates remove the need for continuous cell culture and maximise cell recovery of pre-plated cells, saving time and resources and offering greater flexibility for assay development and toxicological analysis.

Materials and Methods

ASSAY READY HepG2 plates were produced by plating HepG2 cells in 24- and 96- well plates, at multiple cell densities, and cryopreserving them 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.

Post-thaw cell recovery was calculated by comparing cell counts before cryopreservation and 24 hours post-thaw. Growth curves were obtained by counting cells every 24 hours until 80% confluent. Cell counting was completed by cell detachment with trypsin, Trypan blue staining (0.4%) and using a haemocytometer for counting. Membrane integrity was assessed by: (1) measuring lactate dehydrogenase (LDH) release into cell culture medium 24 hours after cryopreserving and intracellular LDH content using the CyQUANT™ LDH Cytotoxicity Assay kit (Thermo Fisher Scientific) and (2) staining cells with AM-Calcein and Ethidium Iodide provided by the LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells (Thermo Fisher Scientific). Metabolic activity was measured using a resazurin reduction assay, by monitoring the conversion of resazurin to resorufin at absorbance wavelengths of 570 and 600 nm.

Reproducible recovery of cells with minimal well-to-well variation

HepG2 cells derive from a hepatocellular carcinoma and perform many differentiated hepatic functions. Hence, HepG2 cells are commonly used in hepatotoxicity studies and drug metabolism. Cellular recovery is crucial to the success of a cryopreservation outcome, providing the user with sufficient cells for experimentation. In addition, minimal well-to-well variability should be observed to ensure consistency in assay measurements. HepG2 cells cryopreserved with CryoShield™ as confluent monolayers in 24- and 96-well plates possess recovery rates of approximately 80%, **Figure 2A**, whereas conventional cryoprotectants (10% DMSO) are unable to achieve high recovery values. The values observed are comparable to suspension cryopreserved cells, with the advantage of being in the format for immediate use with no additional culture. In addition, HepG2 cells retain their adherent, healthy morphology 24 hours post-thaw, **Figure 2B**.

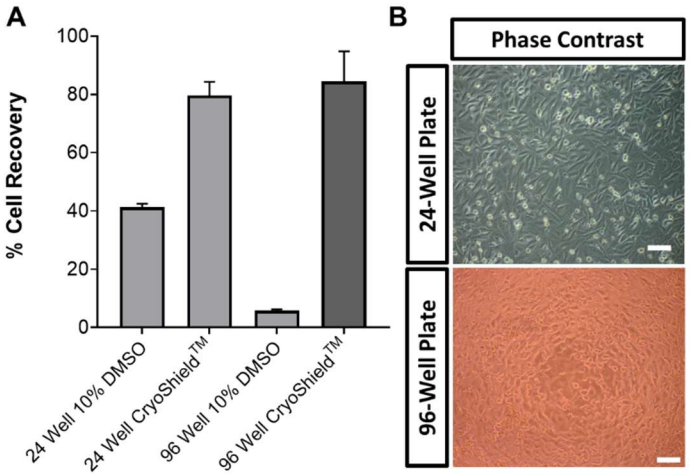

Figure 2. Recovery of cryopreserved HepG2 monolayers. HepG2 cells were seeded as confluent monolayers, 300k cells/well in 24 well plates and 50k cells/well in 96 well plates, and cryopreserved with either 10% DMSO or CryoShield™. (**A**) Percentage cell recovery was determined by counting cells immediately before cryopreserving and 24 hours post-thaw, to account for cryopreservation-induced delayed-onset cell death. (**B**) Phase contrast images of CryoShield™ cryopreserved HepG2 cells 24 hours post-thaw to visualise healthy and adherent morphology.

Confluent monolayers are useful for imaging-based assays and assays that require a high signal output. However, cell viability assays used in drug toxicity experiments, along with many other assays, require a linear output between cell density and the response (fluorescence, luminescence, absorbance, etc.) so lower cell densities are used. Cryopreservation of lower cell densities adhered to tissue culture plastic is a huge challenge, however, CryoShield™ allows the cryopreservation of cells ranging from low-to-high seeding densities to produce ASSAY READY HepG2 plates for many applications, with cell recovery rates of above 70% at all seeding densities tested, **Figure 3**.

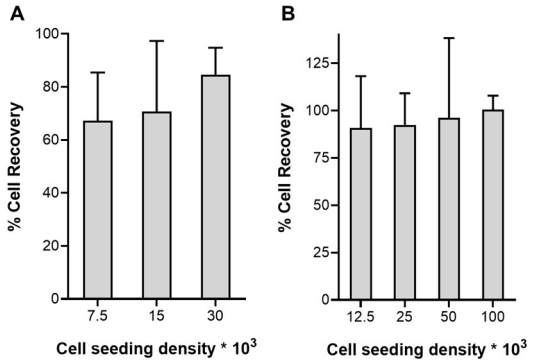

Figure 3. Cryopreserved HepG2 monolayers at different seeding densities. HepG2 cells were cryopreserved in (**A**) 24- and (**B**) 96-well plates at different seeding densities with CryoShield™. Percentage cell recovery was determined 24 hours post-thawing.

Minimal well-to-well variation was observed in cell recovery values, **Figure 4**, a problem commonly encountered in routine cell culture. Minimising cell density variations between wells across well plates reduces the risks of assay variability and, thus, the potential for false positive and negative results.

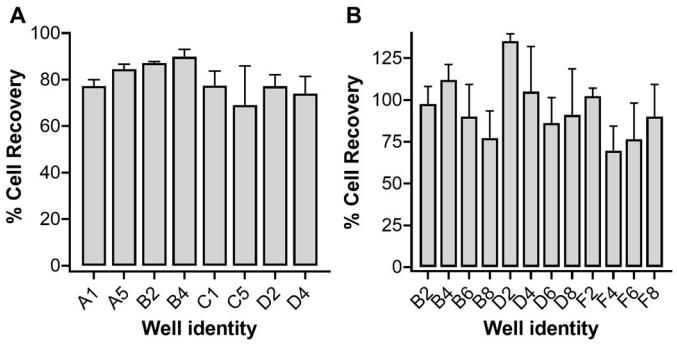

Figure 4. Well-to-well variation in Assay Ready HepG2 plates. The percentage cell recovery of confluent HepG2 monolayers from a selection of wells from (**A**) 24 well plates and (**B**) 96 well plates.

CryoShield™ cryopreserved HepG2 cells retain healthy adherent cellular morphology and intact membranes

CryoShield™ cryopreserved HepG2 cells were thawed and stained with calcein and ethidium iodide after 24 hours, a stain typically used in assays to measure overall cell health (live/dead) based on intact membranes. Healthy adherent cell morphology was observed in cells thawed from both 24- and 96-well plates, **Figure 5A**, with a large portion of cells displaying green, intact membranes as opposed to red, membrane damaged cells. Approximately 80% of cells retained intact membranes, **Figure 5B,** comparable to the cell recovery measurements obtained.

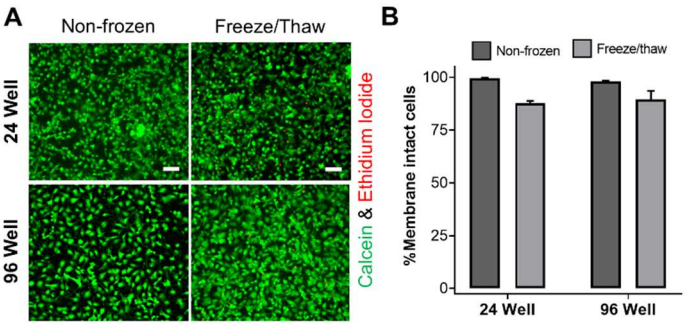

Figure 5. Calcein/ ethidium iodide staining. (**A**) HepG2 cells in cryopreserved with CryoShield™, thawed for 24h and stained with calcein (green, intact membranes) and ethidium iodide (red, damaged membranes). Non-frozen, conventionally cultured cells stained with calcein and ethidium iodide provided for comparison. (**B**) The percentage of membrane intact cells.

Membrane integrity is a crucial assessment for the health of hepatocytes as enzyme leakage would be detrimental to enzyme activity assays used in hepatotoxicity screening for the identification of bioactive drugs and their potential mechanisms. To further assess potential enzyme leakage from CryoShield™ cryopreserved HepG2 cells, the quantity of lactate dehydrogenase (LDH) released from cell populations after freeze/thaw (spontaneous release), compared to non-frozen cells, and the intracellular LDH content (maximum release) within cells was measured, **Figure 6**. No LDH was spontaneously released into the cell culture medium after freeze-thaw and minimal differences were observed between the intracellular content of LDH between non-frozen and frozen HepG2 cells.

Thus, Cryoshield™ cryopreserved HepG2 cells possess the necessary enzymes required for drug metabolism studies and hepatotoxicity screening.

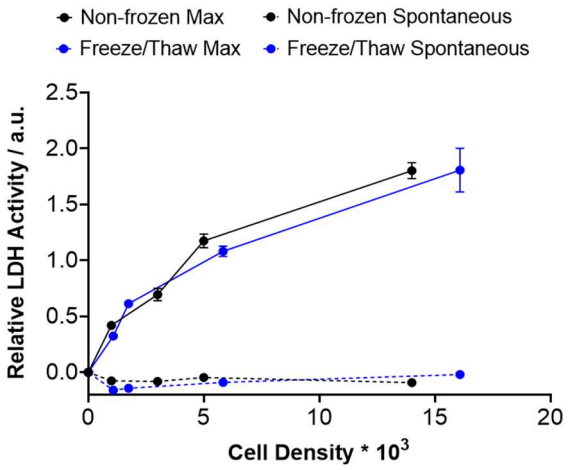

Figure 6. Lactate dehydrogenase (LDH) release. HepG2 cells cryopreserved with CryoShield™ were thawed for 24 hours and LDH released into the cell culture medium (spontaneous) was quantified. The intracellular LDH content (Max) was also assessed. LDH measurements for non-frozen cells were also taken for comparison.

ASSAY-READY HepG2 cells proliferate immediately post-thaw and retain metabolic activity

HepG2 cells retained normal proliferative capacity immediately following thawing, confirming their viable and functional state. HepG2 cells can have a doubling time of 48 hours, however on collagen coated matrixes the doubling time can decrease to 24 hours, **Figure 7A**. Metabolic activity assays, such as the resazurin reduction assay, are another cell health indicator used frequently to assess cell viability in drug assays and require a linear relationship between the number of cells present and absorbance/ fluorescence output. PubMed lists over 200 publications citing resazurin and cancer research and over 1,000 publications in drug screening, development and discovery. A resazurin reduction assay on HepG2 cells at different cell densities revealed that metabolic activity is restored to normal levels 24 hours after thawing cells, **Figure 7B**. A linear response was attained between cell seeding density and percentage resazurin reduction, confirming that ASSAY-READY HepG2 cells can be used for crucial assays used in drug testing.

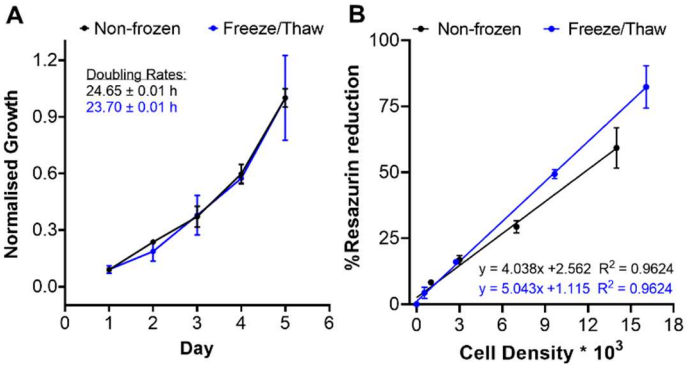

Figure 7. HepG2 proliferative capacity and metabolic activity. (**A**) HepG2 cells were counted daily after thawing for 5 days to determine growth rates. (**B**) A resazurin reduction assay was completed on HepG2 cells cryopreserved at different cell densities to confirm recovery of metabolic activity and a linear response for drug testing.

Conclusion

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. ASSAY READY HepG2 plates are compatible with multiple cell viability assays which are fundamental for drug discovery and hepatotoxicity testing, offering the potential to maximise time spent on research-based activities to transform the field of drug discovery and assay development.