CYP induction and metabolism in plated HepaRG cells prepared with “no-spin” thawing method

Introduction

Gibco™ HepaRG™ cells are a human hepatic progenitor cell line that retains many characteristics of primary human hepatocytes, and has become a predominant *in vitro* research tool since its isolation in 1999. HepaRG cells offered through Thermo Fisher Scientific are terminally differentiated and provided in a convenient cryopreserved format of 10^7 cells/vial (enough for an entire 96-well plate). Since hepatotoxicity is a leading cause for drug failure in clinical trials, finding a reproducible and predictive *in vitro* tool has been imperative for scientists, and hundreds of peer-reviewed manuscripts have since demonstrated the utility of HepaRG cells. Published cytotoxicity studies with HepaRG cells have often focused on drugs and environmental toxicants, such as aflatoxin, which require bioactivation via liver enzymes such as cytochrome P450 enzymes (CYP). Aninat et al. (2006) demonstrated how HepaRG cells form the same CYP-catalyzed toxic epoxide metabolites from aflatoxin as are formed in primary hepatocytes [1]. Guillouzo et al. (2007) built upon this observation by demonstrating aflatoxin toxicity in HepaRG cells, whereas HepG2 cells (which lack CYP activities) were resistant [2].

In addition to metabolism studies, CYP induction is an important study that can be performed *in vitro*, and early in drug development. The latest FDA guidance (2012) states that liver cells maintaining functional AhR, CAR, and PXR can be used for *in vitro* CYP induction studies [3]. Studies have shown that HepaRG cells are responsive to known CYP inducers, demonstrating that these cells maintain functional nuclear transcription factors AhR (CYP1A2), CAR (CYP2B6), and PXR (CYP3A4) (Figure 1). In addition to all of the ADME applications that HepaRG cells can be used for,

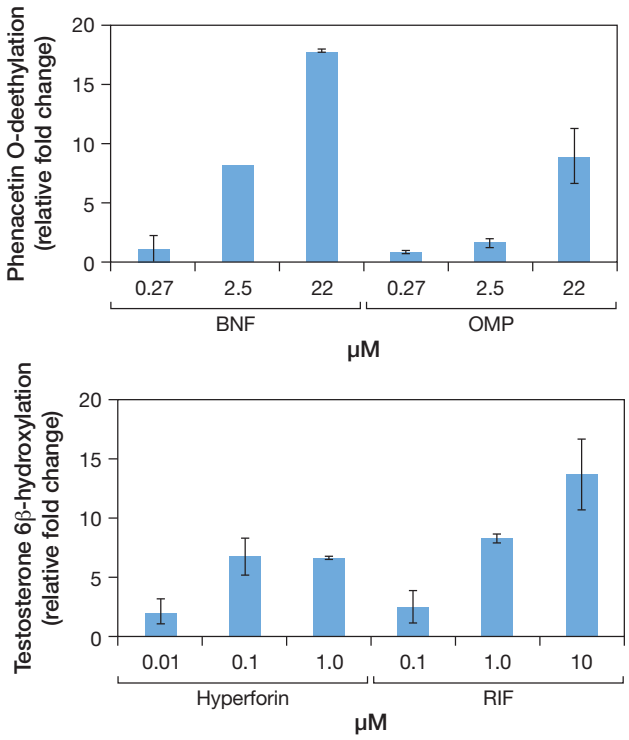

Figure 1. CYP induction in HepaRG cells. HepaRG cells cultured in HepaRG induction medium were treated with different concentrations of known CYP inducers for 72 hours. CYP induction was then assessed by metabolism of probe substrates. BNF: β-naphthoflavone; OMP: omeprazole; RIF: rifampicin.

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these cells have become an attractive alternative to primary hepatocytes because of their lower cost and theoretically unlimited supply. There have been over 200 manuscripts since 1999, from both academic institutions as well as biotech and large pharma.

This growing research community is continuously working on new applications and simplifying the use of these cells, including media supplement recommendations for different applications. The Gibco™ HepaRG™ Induction Media Supplement was optimized to maintain low basal CYP activities for CYP induction research. The Gibco™ HepaRG™ Maintenance/Metabolism Medium Supplement was optimized to increase CYP activities to be more representative to that of primary cryopreserved hepatocytes, for metabolism studies.

A new area of focus for optimization of HepaRG cells is the thawing method. The traditional method of thawing HepaRG cells requires time-consuming centrifugation, cell resuspension, and cell counting prior to plating cells. A newer, more convenient method has been developed as an alternative to the traditional method, which we call the "no-spin" method (Figure 2).

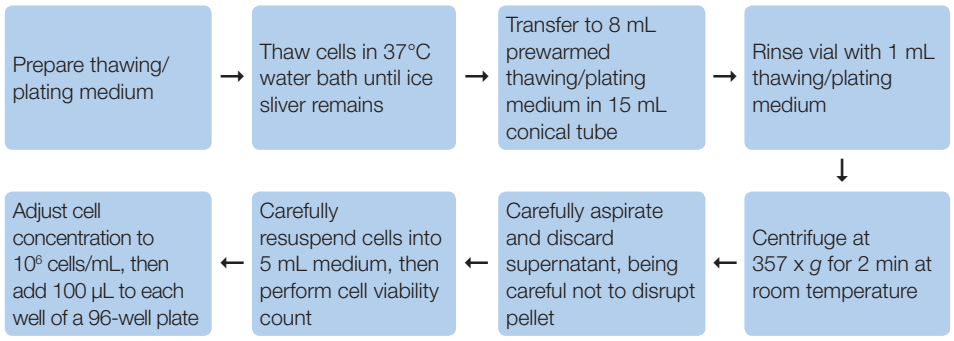
The convenience of not needing to centrifuge, resuspend, and count cells results in less processing time and less mechanical stress to the cells. This results in healthier cells and less user variability in determining cell yield. The "no-spin" thawing method can be used prior to any research application using HepaRG cells. Therefore, this application note was generated to compare the new "no-spin" method with the traditional thawing method in two different types of experiments: (1) using our HepaRG Maintenance/Metabolism Medium Supplement for drug metabolism, and (2) using HepaRG Maintenance/Induction Medium Supplement for CYP induction.

Materials and methods

Thawing HepaRG cells

Base Medium was generated with 99 mL Gibco™ William's E Medium combined with 1 mL Gibco™ GlutaMAX™ Supplement. Gibco™ HepaRG™ Thaw, Plate, & General Purpose Medium Supplement was thawed thoroughly by placing the bottle in a 37°C water bath for 30 minutes. HepaRG Thaw, Plate, & General Purpose Working Medium was then prepared by adding 1 mL HepaRG Thaw, Plate, &

Traditional thawing method



"No-spin" thawing method

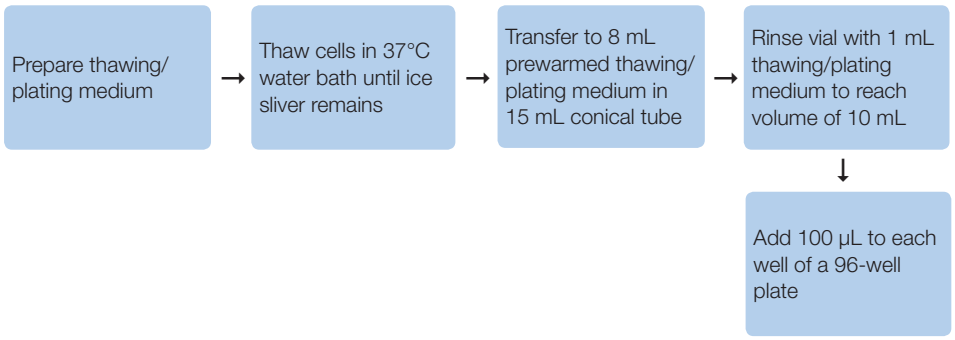


Figure 2. Schematic comparison of traditional thawing and "no-spin" thawing method.

Purpose Medium Supplement to 100 mL Base Medium. HepaRG Thaw, Plate, & General Purpose Working Medium was prewarmed in a 37°C water bath for at least 30 minutes and then pipetted into a sterile 15 mL polystyrene round-bottom tube or similar container. The cryovial was removed from the liquid nitrogen, and quickly transferred to the water bath at 37°C, without allowing water to penetrate into the cap. While holding the tip of the cryovial, the vial was gently agitated just until small ice crystals remained, and then the vial was removed from the water bath. Once the outside of the cryovial was wiped with 70% ethanol absorbent paper, the cryovial was placed under the laminar flow hood. The "semi"-thawed HepaRG cell suspension was aseptically transferred into the tube containing 8 mL of the prewarmed HepaRG Thaw, Plate, & General Purpose Working Medium. The cryovial was rinsed thoroughly once with an additional 1 mL of the HepaRG Thaw, Plate, & General Purpose Working Medium and the resulting suspension was returned to the 15 mL tube. The tube was capped and inverted gently several times to ensure a homogenous suspension of cells was achieved. At this point, one of two procedures were followed:

(1) Standard procedure: Cells were then centrifuged at room temperature at 357 x *g* for 2 min. The supernatant was aspirated and discarded, and the cells were gently resuspended in 5 mL HepaRG Thaw, Plate, & General Purpose Working Medium. Viability and yield was determined using trypan blue, and cells were diluted to 10^6 cells/mL.

For use in 96-well plates, the cells were first transferred from the tube into a reagent reservoir. Using a multichannel pipettor, 100 µL was carefully added directly to each well. The cells were gently agitated in the reservoir often, to keep the cell suspension homogeneous. Once the cells were seeded, the plates were moved to the incubator.

(2) "No-spin" procedure: The centrifuging, resuspending, counting, and dilution steps were skipped, and the cells were used directly, without dilution, for pipetting into 96-well plates. With this improved thawing protocol, there was no need to perform a cell yield count—if the vial was rinsed appropriately with 1 mL media, there will be 10 mL total, and one can assume that there are 10^7 cells in the suspension at a concentration of 10^6 cells/mL.

Application 1: CYP induction

Six hours after the initial cell plating, the existing medium was removed gently from the wells, and 100 µL of the prewarmed working HepaRG Thaw, Plate, & General Purpose Medium Supplement was added to the sides of each well with a multichannel pipette. The plates were then placed back in the 37°C incubator. CYP enzyme activities are known to drop to a lower basal activity over the first 48 hours in culture [4]. Therefore, the medium was simply replenished with new working HepaRG Thaw, Plate, & General Purpose Medium Supplement each day, for the first 2 days in culture. On day 3, HepaRG Induction Working Medium was prepared by adding 1 mL HepaRG Serum-free Induction Supplement to 100 mL Base Medium. HepaRG Induction Working Medium was prewarmed in a 37°C water bath for at least 30 minutes prior to use. Known CYP inducers omeprazole (CYP1A2) and rifampicin (CYP3A4) were solubilized at 1,000X in DMSO, then diluted in HepaRG Induction Working Medium in order to

Table 1. Materials used in these experiments.

Product name	
Collagen I Coated Plate, 96-Well (5 plates)	A1142803
William's E Medium (1X) without Phenol Red	A1217601
GlutaMAX I Supplement	35050061
HepaRG Thaw, Plate, & General Purpose Medium Supplement	HPRG770
HepaRG Serum-free Induction Medium Supplement	HPRG750
HepaRG Maintenance/Metabolism Medium Supplement	HPRG720
Additional materials	
Water bath at 37°C	
Laminar flow hood	
Pipet-Aid™ device, pipettes, and micropipettes	
Multichannel pipettes and repeater pipette	
Polystyrene round-bottom tubes (15 mL)	
Incubator at 37°C with a 5%/95% CO_2/ambient atmosphere and 100% relative humidity	
Phase-contrast microscope	
Material for cell count (cell counting chamber, coverslips, 0.05% trypan blue solution)	

have a final concentration of 0.1% DMSO (Table 2). The cells were treated for a total of 72 hours, replenishing with fresh medium containing inducing compounds each day. Cells from both groups were then incubated with the CYP substrates listed in Table 3 at 37°C while mixing on an orbital shaker. Samples were collected and stored frozen at –70°C until they were processed for LC-MS/MS analysis. Metabolite formation was measured by standard biochemical assays using GLP-validated LC-MS/MS assays.

Application 2: CYP metabolism

HepaRG Maintenance/Metabolism Working Medium was prepared by adding 1 mL HepaRG Maintenance/Metabolism Medium Supplement to 100 mL Base Medium. HepaRG Maintenance/Metabolism Working Medium was prewarmed in a 37°C water bath for at least 30 minutes prior to use. Five hours after the initial plating, the existing medium was removed gently from each well, and 100 µL of the prewarmed HepaRG Maintenance/Metabolism Working Medium was added to the sides of each well with a multichannel pipette. The plates were then placed back in the 37°C incubator. Medium was replaced daily with fresh prewarmed HepaRG Maintenance/Metabolism Working Medium each day until day 7, at which time cells were incubated under the same conditions listed previously in Table 3. Samples were collected and stored frozen at –70°C until they were processed for LC-MS/MS analysis. Metabolite formation was measured by standard biochemical assays using GLP-validated LC-MS/MS assays. Additionally, photomicrographs were taken of the "no-spin" and traditional thawing groups of cells on day 7.

Results

CYP induction

The basal CYP1A2 activities from cells from both the "no-spin" and traditional thawing procedures were not significantly different (Figure 3). However, the induced activities, and therefore the fold-over-control (FOC) values showed a trend and were higher in the "no-spin" cells. Similar effects were observed with CYP3A4. There was no difference detected in the basal CYP3A4 activities; however, the FOC was higher for "no-spin" cells, at 27 FOC, compared with 13 FOC for traditionally plated cells.

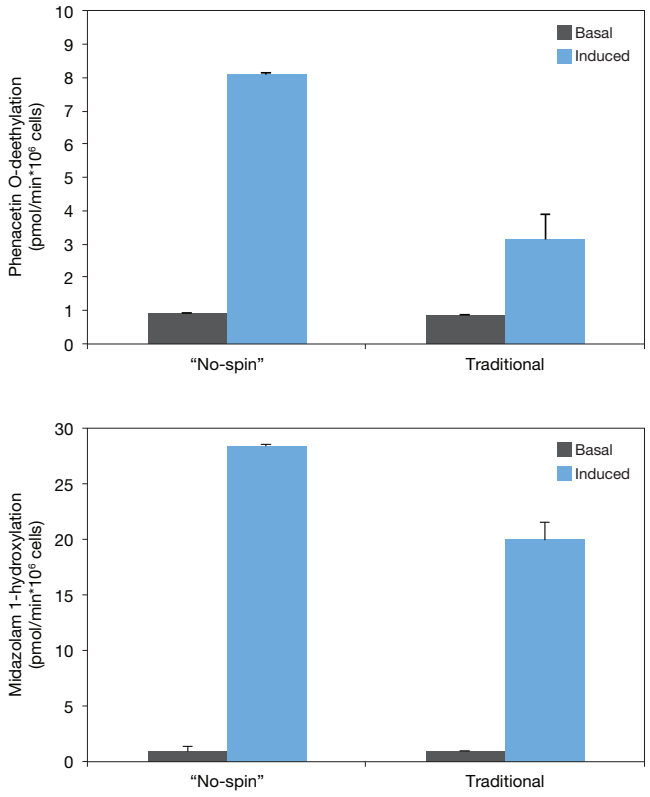


Figure 3. Comparison of CYP1A2 and CYP3A4 induction using the standard and "no-spin" thawing methods. Black bars represent vehicle control treatment groups, and blue bars represent induced treatment groups.

Table 2. CYP inducer conditions.

Inducer	Final conc.	CYP enzyme
Omeprazole	50 µM	CYP1A2
Rifampicin	10 µM	CYP3A4

Table 3. CYP1A2 and CYP3A4 substrates and conditions.

Substrate	Final conc.	Incubation time	CYP enzyme	Metabolite measured
Phenacetin	100 µM	15 min	CYP1A2	APAP
Midazolam	200 µM	14 min	CYP3A4	Hydroxymidazolam

CYP metabolism

Photomicrographs of the “no-spin” and traditional thawing groups of cells appeared indistinguishable (Figure 4). As for enzymatic function, both CYP1A2 and CYP3A4 were significantly more active in cells prepared using the “no-spin” method over the standard thawing method (Figure 5).

Conclusion

The goal of this application note was to determine whether eliminating the centrifugation/resuspension steps from the traditional thawing method would be detrimental to HepaRG cells and downstream assays. The results of these current studies demonstrate that the cells from the “no-spin” protocol were as healthy as cells thawed with the traditional method. In fact, the “no-spin” cells consistently performed with higher metabolic capabilities than those cells from the traditional thawing method. The “no-spin” cells are likely healthier and more functional because they are not subjected to the mechanical stress during manipulations of being centrifuged or resuspended. Furthermore, the viability and attachment efficiency of the “no-spin” cells are likely greater because these cells spend less time in suspension before plating (1–5 min) than traditionally thawed cells (10–20 min, depending on the researcher). In conclusion, the “no-spin” thawing method can be used for all HepaRG applications, resulting in a more convenient, healthier, and scalable system.

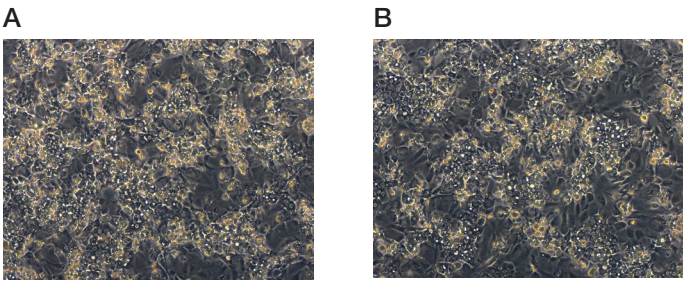


Figure 4. Photomicrographs of cells plated with the “no-spin” method and the traditional method. (A) “No-spin” thawing method. **(B)** Traditional thawing method. Both were plated at the recommended density of 10^5 cells/well.

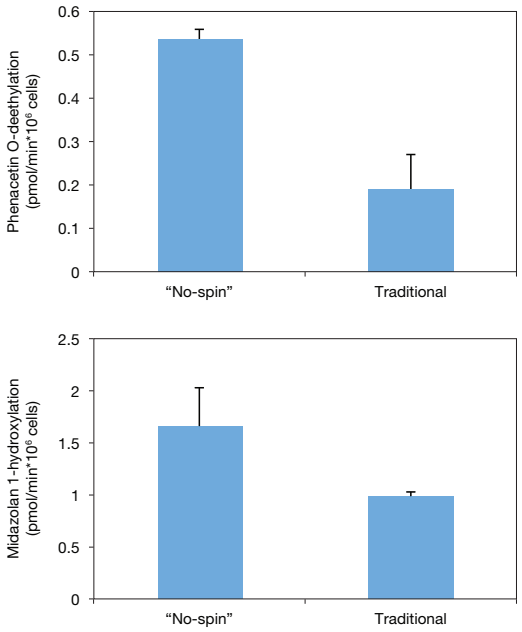


Figure 5. Comparison of CYP1A2 and CYP3A4 metabolism using the standard and “no-spin” thawing methods. Data represented in pmol/min per 10^6 cells.

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References

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