HepaRG™ Cell User Guide

Background

HepaRG™ cells are a human hepatic progenitor cell line that retains many characteristics of primary human hepatocytes. HepaRG™ cells are terminally differentiated and provided in a convenient cryopreserved format. For scientists who need reproducible metabolism data, HepaRG™ cells are an **in vitro** tool that provides reproducible results in a metabolically complete and scalable system.

This description and user guide for the thawing and culture of cryopreserved HepaRG™ cells includes three sections:

→ **Section 1:** Recommended materials, media and cells

→ **Section 2:** Protocol for the thawing, seeding and maintenance of HepaRG™ cells

→ **Section 3:** Cell morphology

→ **Section 4:** Media volume tables

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02.13.2012

Section 1: Materials, media and cells

Materials

- Water bath at +37°C
- Laminar flow hood
- Pipet-aid, pipettes and micropipettes
- Multichannel pipettes and repeater pipette
- Polystyrene round-bottom tubes (40mL) and petri dishes (92 x 17 mm) or similar containers
- 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)

Plates

Catalog #	Description
A1142802	Collagen I, Coated Plate 24-Well
A1142803	Collagen I, Coated Plate 96-Well

Media supplements

For use with 100 ml of William's Medium E

Catalog #	Description
HPRG620	HepaRG™ Maintenance/Metabolism Medium Supplement
HPRG630	HepaRG™ Tox Medium Supplement
HPRG640	HepaRG™ Induction Medium Supplement
HPRG650	HepaRG™ Serum-free Induction Medium Supplement
HPRG670	HepaRG™ Thaw, Plate, & General Purpose Medium Supplement

For use with 500 ml of William's Medium E

Catalog #	Description
HPRG720	HepaRG™ Maintenance/Metabolism Medium Supplement (5x)
HPRG730	HepaRG™ Tox Medium Supplement (5x)
HPRG740	HepaRG™ Induction Medium Supplement (5x)
HPRG750	HepaRG™ Serum-free Induction Medium Supplement (5x)
HPRG770	HepaRG™ Thaw, Plate, & General Purpose Medium Supplement (5x)

- Working medium is prepared by adding the HepaRG™ Supplement to 100 ml/500 ml of Williams' Medium E and 1ml/5ml of GlutaMAX™-I (35050061).

Catalog #	Description
12551032	Williams' Medium E (1X), liquid
A1217601	Williams' Medium E (1X) without Phenol Red
35050061	GlutaMAX™-I Supplement

Cells

- Immediately place the cryovial(s) in liquid nitrogen upon receipt

Section 2: Protocol

Note: Observe universal precautions when handling HepaRG™ cells and treat all biologic material as potentially infectious.

The following steps must be performed under a laminar flow hood.

1. Medium preparation

- Base Medium consists of 99 mL/495 mL of Williams' Medium E combined with 1 mL/5 mL of GlutaMAX™ I
- Thaw the HepaRG™ Supplement by placing the bottle in a +37°C water bath until completely thawed.
- Prepare the HepaRG™ working medium by adding the entire contents of the bottle of supplement to 100 mL/500 mL of Base Medium.
- The HepaRG™ working medium is now ready for use. It should be stored at +4°C for a maximum of one month.

  Note: If completing less than 500 mL of media, see Section 4: Media volume tables at the end of the protocol for exact amount of supplement.

2. Thawing and counting of cryopreserved, HepaRG™ Cells (Day 0)

2.1. Thawing

- Pre-warm working HepaRG™ Thaw, Plate, & General Purpose Working Medium in a +37°C water bath.
- Pipet 9 mL (per HepaRG™ cryovial to be used) of pre-warmed HepaRG™ Thaw, Plate, & General Purpose Working Medium into a sterile 40mL polystyrene round-bottom tube or similar container.
- Prepare an absorbent paper with 70 % ethyl alcohol.
- Remove the cryovial from the liquid nitrogen.
- Under the laminar flow hood, briefly twist the cryovial cap a quarter turn (do not open the cryovial completely) to release internal pressure, and then close it again.
- Quickly transfer the cryovial to the water bath at +37°C. Do not submerge it completely; be careful not to allow water to penetrate into the cap. While holding the tip of the cryovial, gently agitate the vial for 1 to 2 minutes (small ice crystals should remain when the vial is removed from the water bath).
- Wipe the outside of the cryovial with the 70% ethyl alcohol absorbent paper, and place the cryovial under the laminar flow hood.
- Aseptically transfer the "semi"-thawed HepaRG™ cell suspension into the tube containing 9 mL of the pre-warmed HepaRG™ Thaw, Plate, & General Purpose Working Medium (resulting in a 1:10 ratio of cell suspension to total volume).

- Rinse out the cryovial once with approximately 1 mL of the HepaRG™ Thaw, Plate, & General Purpose Working Medium and return the resulting suspension to the 40 mL tube.
- Centrifuge the HepaRG™ cell suspension for 2 minutes at 360 g (room temperature).
- Aspirate the supernatant and resuspend the HepaRG™ cell pellet with 5 mL of HepaRG™ Thaw, Plate, & General Purpose Working Medium

2.2. Cell viability and counting

- Pipet 50 µL of a 0.05% Trypan blue solution into one, 1 mL polystyrene round-bottom tube.
- Homogenize the HepaRG™ cell suspension with gentle manual swirling. Then, take 50 µL of this suspension and add it to the tube containing the Trypan blue solution (1/2 dilution).
- Gently homogenize the resulting cell suspension by manual swirling. Take an aliquot and introduce it into a cell counting chamber (e.g., hemocytometer or the Countess™ Automated Cell Counter).
- Perform cell observation and count under the microscope. Living cells exclude the dye while dead cells take it and appear blue. Count the living and dead cells and then calculate cell viability and concentration.

3. Use of HepaRG™ cells

3.1. Metabolism studies: use of HepaRG™ cells in suspension

- After thawing and counting of HepaRG™ cells (Sec 2), cells can be used for metabolism studies in suspension according to your standard protocol using human hepatocytes; however, incubation times may differ from your standard times.
- Incubate the cells with the test substrates according to your protocol for metabolism studies.

Suspension	Day 0	HepaRG™ Thaw, Plate, & General Purpose Working Medium Incubate the cells with the test substrates according to your protocol

3.2. Metabolism studies: Use of HepaRG™ cells in monolayer

3.2.1. Cell seeding

- After the thawing and the counting of HepaRG™ cells (Sec 2), and using the HepaRG™ Thaw, Plate, & General Purpose Working Medium, seed the HepaRG™ cells into a flat-bottom multi-well plate according to the table below:

Plate Format	Number of viable cells per well ($x10^6$)	Volume per well (mL)	Cell concentration ($x10^6$/mL)
24-well plate	0.60	0.50	1.20
96-well plate	0.10	0.10	1.00

- Pre-wet 96-well plate with 45 uL of HepaRG™ Thaw, Plate, & General Purpose Working Media
- Add 80 ul of cell suspension (1.25 X 10^6 cells/mL)
- Wait for 10 min
- Move to incubator
- Place the plate(s) in the incubator at +37°C with a 5%/95% CO_2/Ambient atmosphere and 100% relative humidity.

3.2.2. Cell maintenance for metabolism studies

You have two options:

- Cells can be used immediately after thawing, or following at least 3 days of culture. HepaRG™ cells retain a high level of CYP activities during the first 24 hours following thaw and plating, and these activities then decrease while the cells reconstitute the monolayer, then the activities return during the fourth day in culture, peaking at Day 7-10.

At day 0, 4 hours after plating

- Four hours after plating observe cell morphology under phase-contrast microscope, and when possible, take photomicrographs.
- Cells can be used for metabolism studies according to your standard protocol using human hepatocytes; however, incubation times may differ from your standard times.
- Incubate the cells with the test substrates according to your protocol for metabolism studies. **Note:** Incubation times may need adjustment.

Monolayer	4 hours after plating, Day 0	Thaw and seed the cells using HepaRG™ Thaw, Plate, & General Purpose Working Medium Four hours after plating, incubate the cells with the test substrates according to your protocol

Day 4-Day 7

- One day after thawing, observe cell morphology under phase-contrast microscope, and when possible, take photomicrographs.
- Change from the HepaRG™ Thaw, Plate, & General Purpose Working Media to the HepaRG™ Maintenance/Metabolism Working Medium.
- Pre-warm the HepaRG™ Maintenance/Metabolism Working Medium in a sterile container (12 mL/24 well plate, 9.6 mL/96 well plate) at room temperature.
- Transfer the HepaRG™ Maintenance/Metabolism Working Medium pre-warmed into a 92 x 17 mm Petri dish.
- Remove the lid from the multi-well plate.
- Remove the existing medium from the wells.
- Gently add the pre-warmed HepaRG™ Maintenance/Metabolism Working Medium to the sides of each well with a multichannel pipette (125 µL/well for a 96 multi-well plate and 500 µL/well for a 24 multi-well plate). Do not add the medium directly onto the cells.
- Control visually the medium level in the wells.
- Put the lid back on the multi-well plate and place the plate(s) back in the +37°C incubator.
- Maintain the HepaRG™ cells in HepaRG™ Maintenance/Metabolism Working Medium and use the cells:

At Day 4

- At day 4, after thawing and culture, a cell monolayer can be observed with a hepatocyte-like cell organization in clusters and metabolic activities are lower than activities detected at day 7-10.

<table>
<tr><td rowspan="3">Monolayer
Day 4</td><td>Day 0</td><td>Thursday</td><td>Thaw and seed the cells using HepaRG™ Thaw, Plate, & General Purpose Working Medium</td></tr>
<tr><td>Day 1</td><td>Friday</td><td>Remove Thaw and seed the cells using HepaRG™ Thaw, Plate, & General Purpose Working Medium, and replace with the HepaRG™ Maintenance/Metabolism Working Medium</td></tr>
<tr><td>Day 4</td><td>Monday</td><td>Incubate the cells in monolayer with the test substrates according to your protocol</td></tr>
</table>

At Day 7

- For optimal activity levels, HepaRG™ Maintenance/Metabolism Working Medium must have been renewed at Day 4 and Day 6.
- After 7-10 days in culture, cells are organized in well-delineated trabeculae with many bright canaliculi-like structures and peak basal metabolic activity.

<table>
<tr><td rowspan="5">Monolayer
Day 7</td><td>Day 0</td><td>Thursday</td><td>Thaw and seed the cells using HepaRG™ Thaw, Plate, & General Purpose Working Medium</td></tr>
<tr><td>Day 1</td><td>Friday</td><td>Remove HepaRG™ Thaw, Plate, & General Purpose Working Medium, and replace with the HepaRG™ Maintenance/Metabolism Working Medium</td></tr>
<tr><td>Day 4</td><td>Monday</td><td>Renew the HepaRG™ Maintenance/Metabolism Working Medium</td></tr>
<tr><td>Day 6</td><td>Wednesday</td><td>Renew the HepaRG™ Maintenance/Metabolism Working Medium</td></tr>
<tr><td>Day 7</td><td>Thursday</td><td>Incubate the cells in monolayer with the test substrates according to your protocol</td></tr>
</table>

3.3. Induction studies

3.3.1. Cell Seeding

- After the thawing and the counting of HepaRG™ cells (Sec 2), and using the HepaRG™ Thaw, Plate, & General Purpose Working Medium, seed the HepaRG™ cells into a flat-bottom multi-well plate according to the table below:

Plate Format	Number of viable cells per well ($x10^6$)	Volume per well (mL)	Cell concentration ($x10^6$/mL)
24-well plate	0.60	0.50	1.20
96-well plate	0.10	0.10	1.00

- Pre-wet 96-well plate with 45 uL of HepaRG™ Thaw, Plate, & General Purpose Working Media
- Add 80 ul of cell suspension (1.25 X 10^6 cells/mL)
- Wait for 10 min
- Move to incubator
- Place the plate(s) in the incubator at +37°C with a 5%/95% CO_2/Ambient atmosphere and 100% relative humidity

3.3.2. Culture and maintenance for induction study

- Six hours after plating (see the suggested timeline), observe cell morphology under phase-contrast microscope, and when possible, take photomicrographs.
- Renew the HepaRG™ Thaw, Plate, & General Purpose Working Medium
- Pre-warm the HepaRG™ Thaw, Plate, & General Purpose Working Medium into a sterile container (12 mL/24 well plate, 9.6 mL/96 well plate) at room temperature.
- Transfer pre-warmed HepaRG™ Thaw, Plate, & General Purpose Working Medium into a 92 x 17 mm Petri dish or similar flat-bottom container suitable for use with multichannel pipetors.
- Remove the lid from the multi-well plate.
- Remove the existing medium from the wells.
- Gently add the pre-warmed HepaRG™ Thaw, Plate, & General Purpose Working Medium to the sides of each well with a multichannel pipette (125 µL/well for 96 multi-well plate and 500 µL/well for 24 multi-well plate). Do not add the medium directly onto the cells.
- Control visually the medium level in the wells.
- Put the lid back on the multi-well plate and place the plate(s) back in the +37°C incubator.
- At day 3, observe cell morphology under phase-contrast microscope, and when possible, take photomicrographs.
- Cells can be used for induction studies: choose between two media with:

  No serum: HepaRG™ Serum-free Induction Medium

  Low level of serum: HepaRG™ Induction Medium
- Change from the HepaRG™ Thaw, Plate, & General Purpose Working Medium to either the HepaRG™ Induction Working Medium or HepaRG™ Serum-free Induction Working Medium with the test articles.
- Incubate the cells with the test articles for 48-72hrs (72hrs=peak response)
- Renew the medium with the test articles daily and always with the medium chosen at the beginning of the study

3.3.3. Suggested timeline for induction studies

Day 0	Friday morning	Thaw and seed the cells using HepaRG™ Thaw, Plate, & General Purpose Working Medium
Day 0	Friday end of afternoon (6 h after plating)	Renew the HepaRG™ Thaw, Plate, & General Purpose Working Medium
Day 3	Monday morning	Remove the HepaRG™ Thaw, Plate, & General Purpose Working Medium, and replace with the HepaRG™ Induction Working Medium or HepaRG™ Serum-free Induction Working Medium Incubate the cells in monolayer with the test articles according to your study design. The renewal of the medium with the test articles should be performed daily until Wednesday.
Day 4	Tuesday morning	Renew the HepaRG™ Induction Working Medium or HepaRG™ Serum-free Induction Working Medium
Day 5	Wednesday morning	End of the incubation with the test articles Incubate the cells with the substrate to monitor changes in enzyme activity

3.4. Uptake and transport studies: Use of HepaRG™ cells in suspension

→ After thawing and counting of HepaRG™ cells (Sec 2), cells can be used for uptake and transport studies in suspension according to your standard protocol using human hepatocytes. Incubate the cells with the test substrates according to your protocol for uptake and transport studies; however incubation times may differ from your standard times.

Suspension	Day 0	Thaw and seed the cells using HepaRG™ Thaw, Plate, & General Purpose Working Medium Incubate the cells with the test substrates according to your protocol

3.5. Toxicity studies

3.5.1. Cell seeding

→ After the thawing and the counting of HepaRG™ cells (Sec 2), and using the HepaRG™ Thaw, Plate, & General PurposeWorking Medium, seed the HepaRG™ cells into a flat-bottom multi-well plate according to the table below:

Plate Format	Number of viable cells per well ($x10^6$)	Volume per well (mL)	Cell concentration ($x10^6$/mL)
24-well plate	0.60	0.50	1.20
96-well plate	0.10	0.10	1.00

→ Pre-wet 96-well plate with 45 uL of HepaRG™ Thaw, Plate, & General Purpose Working Media

→ Add 80 ul of cell suspension (1.25 X 10^6 cells/mL)

→ Wait for 10 min

→ Move to incubator

→ Place the plate(s) in the incubator at +37°C with a 5%/95% CO_2/Ambient atmosphere and 100% relative humidity

3.5.2. Culture and maintenance for toxicity study

- → One day after thawing, observe cell morphology under phase-contrast microscope, and when possible, take photomicrographs.
- → Pre-warm the HepaRG™ Tox Working Medium in a sterile container (12 mL/24 well plate, 9.6 mL/96 well plate) at room temperature.
- → Transfer the HepaRG™ Tox Working Medium pre-warmed into a 92 x 17 mm Petri dish.
- → Remove the lid from the multi-well plate.
- → Remove the existing medium from the wells.
- → Gently add the pre-warmed HepaRG™ Tox Working Medium to the sides of each well with a multichannel pipette (125 µL/well for a 96 multi-well plate and 500 µL/well for a 24 multi-well plate). Do not add the medium directly onto the cells.
- → Control visually the medium level in the wells.
- → Put the lid back on the multi-well plate and place the plate(s) back in the +37°C incubator.
- → Maintain the HepaRG™ cells in HepaRG™ Tox Working Medium until the use of cells at day 7.
- → Renew the HepaRG™ Tox Working Medium.

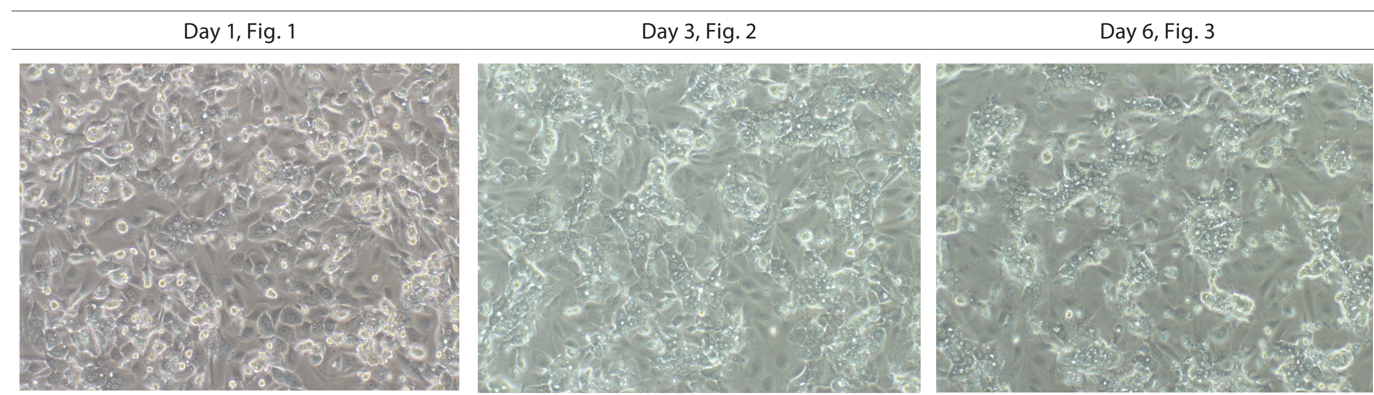
3.5.3. Suggested timeline for toxicity studies

Day 0	Thursday	Thaw and seed the cells using HepaRG™ Thaw, Plate, & General Purpose Working Medium
Day 1	Friday	Remove HepaRG™ Thaw, Plate, & General Purpose Working Medium, and replace with the HepaRG™ Tox Working Medium."
Day 4	Monday	Renew the HepaRG™ Tox Working Medium
Day 7	Thursday	Renew the HepaRG™ Tox Working Medium and incubate the cells in monolayer with the test articles according to your protocol

Section 3: Cell morphology

- After 1 day of culture, hepatocyte-like cells appear in small, differentiated colonies, individualized (fig 1).
- After 3-4 days of culture, a restructuring of cell monolayer can be observed with an hepatocyte-like cells' organization in clusters (fig 2).
- 6-7 days after plating, hepatocyte-like cells are organized in well-delineated trabeculae with many bright canaliculi-like structures (fig 3).

Day 1, Fig. 1	Day 3, Fig. 2	Day 6, Fig. 3



Section 4: Media volume tables

- Actual volumes dispensed are correct as labeled on the vial.
- Minimal extra volume was aliqouted for certain supplements to account for pipetting error.
- If supplementing either 100 or 500 mL of HepaRG Base Media, you can add the entire contents of the bottle without affecting results.

SKU	Volume (mL)
HPRG620	16
HPRG630	14
HPRG640	4
HPRG650	2
HPRG670	14
HPRG720	79
HPRG730	67
HPRG740	19
HPRG750	8
HPRG770	67

SKU	50 mL	100 mL
HPRG620	7.8	15.6
HPRG630	6.7	13.3
HPRG640	1.9	3.7
HPRG650	0.8	1.6
HPRG670	6.7	13.3

SKU	50 mL	100 mL	200 mL	300 mL	400 mL	500 mL
HPRG720	7.8	15.6	31.3	46.9	62.6	78.2
HPRG730	6.7	13.3	26.6	39.9	53.2	66.6
HPRG740	1.9	3.7	7.5	11.2	15.0	18.7
HPRG750	0.8	1.6	3.2	4.8	6.4	8.0
HPRG770	6.7	13.3	26.6	39.9	53.2	66.6

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