USER GUIDE - HepaRG™ Cells

Catalog Number HPR116

Product description

HepaRG™ is an unique and well established hepatic cell system able to produce early hepatic progenitor cells as well as completely mature human hepatocytes.

HepaRG™ cells are more economical, convenient, and predictable than fresh or cryopreserved primary human hepatocytes. It is available in proliferative state to be expanded and differentiated in-house (HPR101), or as cryopreserved, fully differentiated and ready-to-use hepatic cells (HPR116).

LIMITED USE LICENSE

HepaRG™ cells are owned and patented by INSERM and their use is strictly restricted; the Cells are provided under a usage right under which they must be treated as a single-use disposable product that must be destroyed upon conclusion of a study or experiment. Propagating, reproducing, cloning, subcloning, or any use of the Cells following the conclusion of a study, are prohibited. Using the Cells themselves to act as, produce, or manufacture commercial products for sale or intended for sale, is prohibited. Transfer of the Cells, whether for compensation or not, to (i) anyone who is not employed within the same organization, or (ii) who is not involved in a formally-established Scientific Collaboration with the original recipient of the Cells, is prohibited. If the Cells are transferred to a Scientific Collaborator, the original recipient of the Cells is obligated to inform the Collaborator of these restrictions and if the recipient becomes aware of any violation of these restrictions by the Scientific Collaborator, to promptly communicate to the Scientific Collaborator to cease the violation(s) and to notify Biopredic of the violation. If a recipient of the Cells is unwilling to accept these terms of this Limited Use License, they should not be ordered, and if received, should be immediately returned in a re-usable state using the same shipping conditions as those used for shipment of the Cells to the recipient. Violations of this Limited Use License will be prosecuted to the fullest extent of the law.

Contents and storage

Contents	Amount	Storage
HepaRG™ Cells	≥8× 10^6 viable cells/vial	Liquid nitrogen

Required materials not included:

Unless otherwise indicated, all materials are available through **saferworldbydesign.com**

Item	Source
Media Supplements (For use with William's E Medium)	
HepaRG™ Thaw, Plate & General Purpose Medium Supplement	ADD671C
HepaRG® Thawing/Plating/General Purpose Medium Supplement with antibiotics	ADD670C
HepaRG™ Maintenance/Metabolism Medium Supplement	ADD621C
HepaRG® Maintenance/Metabolism Medium Supplement with antibiotics	ADD620C
HepaRG® Induction Medium Supplement	ADD641C
HepaRG® Induction Medium Supplement with antibiotics	ADD640C

HepaRG™ Serum-free Induction Medium Supplement	ADD651C
HepaRG® Serum-free Induction Medium Supplement with antibiotics	ADD650C
HepaRG® Differentiation Medium Supplement	ADD721C
HepaRG® Differentiation Medium Supplement with antibiotics	ADD720C
HepaRG® Growth Medium Supplement	ADD711C
HepaRG® Growth Medium Supplement with antibiotics	ADD710C
HepaRG® Pre-induction Medium Supplement	ADD611C
HepaRG® Pre-induction Medium Supplement with antibiotics	ADD610C
Media and supplements	
William's E Medium (1X), liquid	MLS
GlutaMAX™ Supplement	MLS
William's E Medium, GlutaMAX™ Supplement	MLS
Coated cell culture supports	
Collagen I, Coated Plate 6-Well	MLS
Collagen I, Coated Plate 6-Well	MLS
Equipment	
Water bath at +37°C	MLS
Laminar flow hood	MLS
Pipet-aid, pipettes and micropipettes	MLS
Polystyrene round-bottom tubes (40 mL) and petri dishes or other containers	MLS
Incubator at 37°C with 5% CO_2	MLS
Phase contrast microscope	MLS
Nageotte chamber, coverslips, 0.05% Tryptan blue solution	MLS

Media preparation

Prepare HepaRG™ Thaw, Plate & General Purpose Medium

Thaw the HepaRG™ Thaw, Plate & General Purpose Medium Supplement (Cat. Nos. **ADD671C**) by placing the bottle in a +37°C water bath until completely thawed.

- Add the thawed HepaRG™ Thaw, Plate & General Purpose Medium Supplement (Cat. No. **ADD671C**) to 100 mL of William's E Medium and 1 mL GlutaMAX™ Supplement, or 100 mL William's E Medium with GlutaMAX™ Supplement.

The working media, HepaRG™ Thaw, Plate & General Purpose Medium, is now ready for use. Store at +4°C for a maximum of one month.

Prepare HepaRG™ Maintenance/Metabolism Medium

Thaw the HepaRG™ Maintenance/Metabolism Medium Supplement (Cat. Nos. **ADD621C**) by placing the bottle in a +37°C water bath until completely thawed.

- Add the thawed HepaRG™ Maintenance/Metabolism Medium Supplement (Cat. No. **ADD621C**) to 100 mL of William's E Medium and 1 mL GlutaMAX™ Supplement, or 100 mL William's E Medium with GlutaMAX™ Supplement.

The working media, HepaRG™ Maintenance/Metabolism Medium, is now ready for use. Store at +4°C for a maximum of one month.

Prepare HepaRG™ Induction Medium Supplement

Thaw the HepaRG™ Induction Medium Supplement (Cat. Nos. **ADD641C**) by placing the bottle in a +37°C water bath until completely thawed.

- Add the thawed HepaRG™ Induction Medium Supplement (Cat. No. **ADD641C**) to 100 mL of William's E Medium and 1 mL GlutaMAX™ Supplement, or 100 mL William's E Medium with GlutaMAX™ Supplement.

The working media, HepaRG™ Induction Medium Supplement, is now ready for use. Store at +4°C for a maximum of one month.

Prepare HepaRG™ Serum-free Induction Medium

Thaw the HepaRG™ Serum-free Induction Medium Supplement (Cat. Nos. **ADD651C**) by placing the bottle in a +37°C water bath until completely thawed.

- Add the thawed HepaRG™ Serum-free Induction Medium Supplement (Cat. No. **ADD651C**) to 100 mL of William's E Medium and 1 mL GlutaMAX™ Supplement, or 100 mL William's E Medium with GlutaMAX™ Supplement.

The working media, HepaRG™ Serum-free Induction Medium, is now ready for use. Store at +4°C for a maximum of one month.

Thaw cells

1. Pre-warm the HepaRG™ Thaw, Plate & General Purpose Medium in the +37°C water bath.
2. Pipet 9 mL (per cryovial of HPR116) of prewarmed HepaRG™ Thaw, Plate & General Purpose Medium into a sterile 40-mL polystyrene round-bottom tube or similar container.

   Prepare an absorbent paper with 70% ethyl alcohol.
3. Remove the cryovial from the liquid nitrogen.
4. Under the laminar flow hood, briefly twist the cap a quarter turn (do not open the cryovial completely) to release the internal pressure, and then close it again.
5. Quickly transfer the cryovial to the water bath at +37°C.

   Do not submerge it completely, being careful not to allow water to penetrate into the cap.
6. While holding the tip of the cryovial, gently agitate the vial for about 2 minutes.

   Small ice crystal should remain when removed from the water bath.
7. Wipe the outside of the cryovial with 70% ethyl alcohol on an absorbent paper and place the cryovial under the laminar flow hood.
8. Aseptically transfer the "semi"-thawed HepaRG™ cell suspension into the tube containing 9 mL of the pre-warmed HepaRG™ Thaw, Plate & General Purpose Medium (resulting in a 1:10 ratio of cell suspension to total volume).
9. Rinse out the cryovial once with approximately 1 mL of the HepaRG™ Thaw, Plate & General Purpose Medium and return the resulting suspension to the 40-mL tube.
10. Centrifuge the differentiated HepaRG™ cell suspension 3 min at 500 × g at room temperature.

    Do not use a traditional hepatocyte centrifugation protocol because HepaRG™ cells are smaller in size and need a longer and faster centrifugation.
11. Aspirate the supernatant. To avoid aspiration of cells, leave a little volume of medium on the pellet.
12. Gently resuspend the differentiated HepaRG™ cell pellet in 5 mL of HepaRG™ Thaw, Plate & General Purpose Medium.

    Do not try to dissociate the bigger clusters.

Determine cell viability and count cells

1. Transfer 900 µL Trypan Blue Solution (0.05% in DPBS 1X) in a 5-mL polystyrene round bottom tube.
2. Prepare a cell counting chamber. Carefully clean the counting chamber surface with lens paper.

IMPORTANT!

Use a Nageotte chamber or an appropriate counting cell. Do not use a Malassez cell which is not adapted to cells >10µm.

   Clean the coverslip. Place the coverslip over the counting surface prior to putting on the cell suspension.
3. Gently homogenize the cell suspension by manual swirling.
4. Introduce approximately 100 µL of cell suspension between the mirror-like polished surface and the coverslip.
   The area under the coverslip fills by capillary action. Enough liquid should be introduced so that the mirrored surface is just covered.
5. Observe under a microscope and count living and dead cells on at least four rows distributed throughout the cell counting chamber.
   Living cells exclude the dye, dead cells take up the dye and appear blue. If the total number of cells is quite different from one row to another, count one or two more rows.

IMPORTANT!

Thawed HepaRG™ cells can form clusters. It is necessary to count all the cells including those forming the clusters.

6. Determine the average number of viable cells and dead cells per row.
7. Determine percentage of cell viability.

$$\frac{\text{Number of viable cells} \times 100}{\text{Number of viable cells} + \text{Number of dead cells}}$$

8. Calculate the cell concentration in million cells / mL.
   Sample calculation with a Nageotte chamber:
   Number of viable cells per row x 10 (dilution factor in Trypan Blue) x 800 (parameter related to Nageotte cell) = M cell/ml.
9. Calculate the total viable cell number:
   Cell concentration in million cells/ml xTotal volume of cell suspension = Total number of cells

Metabolism studies:

1. Use HepaRG™ cells in suspension

Thaw the cells in HepaRG™ Thaw, Plate & General Purpose Medium.

Incubate the cells with the test substrates according to your protocol for metabolism studies.

2. Use HepaRG™ cells in monolayer

Seed HepaRG™ cells

Following cell thawing and counting, seed HepaRG™ cells into flat bottom multi-well plate(s) or flask(s) using HepaRG™ Thaw, Plate & General Purpose Medium according to the table below:

Table 1 Cryopreserved differentiated HepaRG™ cell seeding density

Cell culture support	Number of viable cells per well	Volume per well/flask	Cell concentration
25 cm2 flask	5.2×10^6	5 mL	1.04×10^6/mL
6-well plate	2×10^6	2 mL	1×10^6/mL
12-well plate	0.8×10^6	1 mL	0.8×10^6/mL
24-well plate	0.48×10^6	0.5 mL	0.96×10^6/mL
48-well plate	0.16×10^6	0.2 mL	0.8×10^6/mL
96-well plate	0.072×10^6	0.1 mL	0.72×10^6/mL
384-well plate	0.024×10^6	0.04 mL	0.60×10^6/mL

Procedural guidelines to cell seeding

- If a 96-well plate is partially seeded, fill the wells surrounding those containing the cells with sterile water.
- To avoid "edge effect" (evaporation of medium from wells)in 384-well plates, fill outer rows with 50 µL water. For an even distribution of cells in the wells, avoid causing air bubbles during cell seeding. This can be accomplished by first dispensing 20 µL medium, spinning the plate at 1500 × g for 5 minutes, and then adding 20 µL of cell suspension twice concentrated compared to the value given in the table above (e.g. 20 µL at 1.2×10^6 cells/mL to reach a final concentration of 0.6×10^6 cells/mL for a 384-well plate). Avoid having volumes <35 µL (0.035 mL)or >50 µL (0.05 mL) in any well containing cells.
- Except for the 96- and 384-well plates, gently agitate the supports in a north-south and east-west motion then control under microscope the even distribution of cells.
- Place the plate(s) or flask(s) in the incubator at +37°C, 5% CO2 and saturating humidity

Cell maintenance for metabolism studies

Use the cells immediately after thawing or following at least 3 days of culture. HepaRG™ cells keep a high level of CYP activities during the first 24 hours following thawing and plating. These activities decrease as the cells reconstitute the monolayer and then regain gradually to reach a peak on Day 8

Use the cell monolayer at Day 1, 4 hours after plating

Cells can be used for the metabolism studies according to your standard protocol for human hepatocytes.

Day	Hours after plating	Action
Day 1	4 hours	● Thaw and seed the cells using HepaRG™ Thaw, Plate & General Purpose Medium. ● Four hours after plating, observe cell morphology under phase-contrast microscope and when possible, take photomicrographs. ● Incubate the cells with the test substrates according to your protocol.

Use the cell monolayer at Day 5, 96 hours after plating

At day 5 after thawing and culture: a cell monolayer can be observed with hepatocyte-like cell clusters. Metabolic activities are slightly lower than activities measured in fresh cells.

Day	Day of week	Action
Day 1	Thursday	Thaw and seed the cells using HepaRG™ Thaw, Plate & General Purpose Medium.
Day 2 (24 hours)	Friday	Remove HepaRG™ Thaw, Plate & General Purpose Medium, and replace with the HepaRG™ Maintenance/Metabolism Medium.
Day 5 (96 hours)	Monday	Incubate the cells with the test substrates according to your protocol.

Use the cell monolayer at Day 8, 168 hours after plating

For optimal activity levels, HepaRG™ Maintenance/Metabolism Medium must have been renewed at Day 5 and Day 7.

At day 8 after thawing and culture: cells are organized in well delineated trabeculae with many bright canaliculi-like structures and basal metabolic activities similar to fresh cells.

Day	Day of week	Action
Day 1	Thursday	Thaw and seed the cells using HepaRG™ Thaw, Plate & General Purpose Medium.
Day 2 (24 hours)	Friday	Remove HepaRG™ Thaw, Plate & General Purpose Medium, and replace with the HepaRG™ Maintenance/Metabolism Medium.
Day 5 (96 hours)	Monday	Renew the HepaRG™ Maintenance/Metabolism Medium.
Day 7 (144 hours)	Wednesday	Renew the HepaRG™ Maintenance/Metabolism Medium.
Day 8 (168 hours)	Thursday	Incubate the cells with the test substrates according to your protocol.

Note: Cells can be used for the metabolism studies from Day 5 to Day 8 according to your standard protocol for human hepatocytes. They can also be kept in HepaRG™ Maintenance/Metabolism Medium for 1 additional week, with a renewal of this culture medium every 2–3 days.

For 12- 24- 48- 96- 384-well plate(s)

1. Pre-warm the HepaRG™ Maintenance/Metabolism Medium in a sterile container (12 mL/24- or 12-well plate, 9.6 mL/48- or 96-well plate, 8 mL/384-well plate [assuming 200 usable wells], plus a little extra) at room temperature.
2. Transfer the pre-warmed HepaRG™ Maintenance/ Metabolism Medium into a 92 × 17 mm Petri dish or similar flat-bottom container suitable for the use of multichannel pipette.
3. Remove the lid from the multi-well plate.
4. Remove the existing medium from the wells.
5. Gently add the pre-warmed HepaRG™ Maintenance/Metabolism Medium to the sides of each well with a multichannel pipette (for volume per well, see Table 1).

   Do not add the medium directly onto the cells.
6. Control visually the medium level in the wells.
7. Put the lid back on the multi-well plate and place the plate(s) back in the +37°C incubator.

For 6 well plate(s):

1. Pre-warm the HepaRG™ Maintenance/Metabolism Medium in a sterile container (12 mL/6-well plate) at room temperature.
2. Remove the lid from the multi-well plate.
3. Remove the existing medium from the wells.
4. Gently add the pre-warmed HepaRG™ Maintenance/Metabolism Medium to the sides of each well with a pipette (2 mL per well).

   Do not add the medium directly onto the cells.
5. Control visually the medium level in the wells.
6. Put the lid back on the multi-well plate and place the plate(s) back in the +37°C incubator.

For 25 cm2 flask(s)

1. Pre-warm the HepaRG™ Maintenance/Metabolism Medium at room temperature.
2. Remove the cap of the flask(s).
3. Aspirate the existing medium from the flask.
4. Transfer 5 mL of pre-warmed HepaRG™ Maintenance/Metabolism Medium into the 25 cm2 flask.

   Take care not to pipette down the medium directly on the cells.
5. Close and place the flask(s) back in the +37°C incubator.

Induction studies: Use HepaRG™ cells in monolayer

For cell seeding, see "Seed HepaRG™ cells" on page 5.

Culture and maintenance for induction study

1. Six hours after plating (see the suggested timeline), observe cell morphology under phase contrast microscope, and when possible, take photo-micrographs.
2. Renew the HepaRG™ Thaw, Plate & General Purpose Medium (see "Cell maintenance for metabolism studies" on page 6).
3. At day 4, after 72 hours of culture, observe cell morphology under phase-contrast microscope, and when possible, take photomicrographs.
4. Cells can be used for induction studies; choose between media with:
   - No serum: HepaRG™ Serum-free Induction Medium
   - Low level of serum: HepaRG™ Induction Medium
5. Change from the HepaRG™ Thaw, Plate & General Purpose Medium to either the HepaRG™ Serum-free Induction Medium or HepaRG™ Induction Medium with the test substances.
6. Incubate the cells with the test substances for 48 hours.
7. Renew the medium with the test substances daily and always with the medium chosen at the beginning of the study (either HepaRG™ Serum-free Induction Medium or HepaRG™ Induction Medium).

   Note: *Maximal fold induction of metabolic activity may be achieved with 72-hour treatment time, but published data indicate that 48 hours of treatment is sufficient to demonstrate significant induction of CYP1A2, CYP2B6, and CYP3A4 metabolic activity using prototypical inducers.*

   For assessment of enzyme induction by measuring mRNA levels, 24-hour treatment time is frequently used, but 48 hours of treatment will ensure maximum induction (if any).

Suggested timeline for induction studies

Day	Day of week	Action
Day 1	Friday morning	Thaw and seed the cells using HepaRG™ Thaw, Plate & General Purpose Medium.
Day 2 (24 hours)	Saturday	Remove the HepaRG™ Thaw, Plate & General Purpose Medium and replace with the HepaRG™ Maintenance/Metabolism Medium.
Day 4 (72 hours)	Monday morning	1. Remove the HepaRG™ Maintenance/Metabolism Medium, and replace with the HepaRG™ Induction Medium or HepaRG™ Serum-free Induction Medium. 2. Incubate the cells in monolayer with the test substances according to your study design. The renewal of the medium with the test substances should be performed daily until Wednesday.
Day 5 (96 hours)	Tuesday morning	Renew the HepaRG™ Induction Medium or HepaRG™ Serum-free Induction Medium with the test substances.
Day 6 (120 hours)	Wednesday	End of the incubation with the test substance Incubate the cells with the test substrates

Uptake and transport studies: Use HepaRG™ cells in suspension

1. Thaw the cells in HepaRG™ Thaw, Plate & General Purpose Medium
2. Incubate the cell suspension with the test substrates according to your protocol for uptake and transport studies.

Toxicity studies: Use HepaRG™ cells in monolayer

For cell seeding, see “Seed HepaRG™ cells“ on page 5.

Culture and maintenance for toxicity study

1. One day after thawing, observe cell morphology under phase-contrast microscope, and when possible, take photomicrographs.
2. Renew the HepaRG™ Thaw, Plate & General Purpose Medium (see “Cell maintenance for metabolism studies“ on page 6).
3. Maintain the HepaRG™ cells in HepaRG™ Thaw, Plate & General Purpose Medium until the use of cells at day 8.
4. Renew the HepaRG™ Thaw, Plate & General Purpose Medium, and incubate the cells in monolayer with the test substances according to your protocol.

Suggested timeline for toxicity studies

Day	Day of week	Action
Day 1	Thursday	Use HepaRG™ Thaw, Plate & General Purpose Medium to thaw and seed the cells.
Day 2 (24 hours)	Friday	Remove the HepaRG™ Thaw, Plate & General Purpose Medium and replace with the HepaRG™ Maintenance/Metabolism Medium.
Day 5 (96 hours)	Monday	Renew HepaRG™ Maintenance/Metabolism Medium.
Day 7 (144 hours)	Wednesday	Renew HepaRG™ Maintenance/Metabolism Medium.
Day 8 (168 hours)	Thursday	Remove HepaRG™ Maintenance/Metabolism Medium and incubate the cells in monolayer with the test substances according to your protocol.

Cell morphology

- After 24 hours of culture, hepatocyte-like cells appear as small, individualized, and differentiated colonies, (Figure 1).

- After 72–96 hours of culture, a restructuring of cell monolayer can be observed with hepatocyte-like cell clusters (Figure 2).

- 120–144 hours after plating, hepatocyte-like cells are organized in well-delineated trabeculae with many bright canaliculi-like structures (Figure 3).

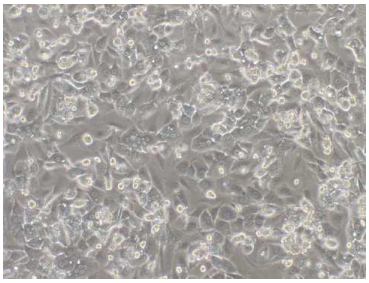

Figure 1

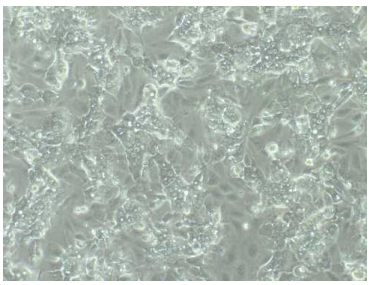

Figure 2

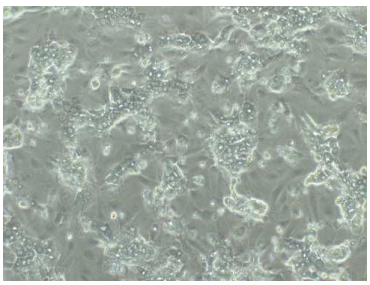

Figure 3