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DIFFERENTIATED HepaRG® CELLS CRYOPRESERVED

Description and user guide
For thawing, culture, and use

Catalog number: HPR116080

Storage Temp -150 °C

BACKGROUND

HepaRG® cells have the unique properties of maintaining significant levels of hepatic cell functions, of being CYP450 inducible, and supporting the complete replicative cycle of HBV.

This description and use guide for the thawing and culture of cryopreserved differentiated HepaRG® includes three sections:

SECTION 1: MATERIALS, MEDIA AND CELLS Error! Bookmark not defined.

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LIMITED USE LICENSE

HepaRG® cells are patented and their use is strictly limited; consider the cells as a single-use, disposable product that must be destroyed upon conclusion of a study or experiment. Propagating, reproducing, cloning, subcloning or any other use of the cells following the conclusion of a study is prohibited. Use of the cells to produce or manufacture commercial products for general sale or for use in the manufacture of products intended for general sale is prohibited. Transfer of the cells to anyone not employed within the same organization, whether for financial benefit or not, is prohibited. **If you are unwilling to accept the terms of this LIMITED USE LICENSE, do not ORDER or use them, and immediately return the cells for credit. Violators of this Limited Use License will be prosecuted to the fullest extent of the law.**

For more information and all publications on HepaRG®, visit

www.HepaRG.com

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SECTION 1: MATERIALS, MEDIA, AND CELLS

1. Materials

- Water bath at +37 °C
- Laminar flow hood
- Pipette-aid, pipettes, and micropipettes
- Multichannel pipettes
- Polystyrene round-bottom tubes (40 mL) and petri dishes (92 × 17 mm) or similar containers
- Incubator at +37 °C, 5% CO_2 and saturating humidity
- Phase-contrast microscope
- Material for cell count (cell counting chamber, coverslips, 0.05% Trypan blue solution)

2. Coated cell culture supports

The following are provided by BIOPREDIC International with HepaRG®, HPR116.

Designation	Reference	Conditions of	
		Shipping Temp	Storage Temp
25 cm² flask coated with collagen I*	FLA125	+4 °C	+4 °C
6-well plate coated with collagen I*	PLA135	+4 °C	+4 °C
12-well plate coated with collagen I*	PLA138	+4 °C	+4 °C
24-well plate coated with collagen I*	PLA137	+4 °C	+4 °C
48-well plate coated with collagen I*	PLA139	+4 °C	+4 °C
96-well plate coated with collagen I*	PLA136	+4 °C	+4 °C

*BPI proprietary coating process to ensure proper seeding and culture of the HPR116
Collagen-coated plates will allow cells to attach faster, usually within three hours; uncoated plates generally require 4-6 hours for attachment to occur.

3. Media

Article	Provider	Reference	Storage Temp
Williams E Medium (w/o glutamine; w/o phenol red)	Sigma-Aldrich	W1878	+4 °C
Ala-Gln 200mM solution	Sigma-Aldrich	G8541	-20 °C

The following supplements are available from Sigma-Aldrich and BIOPREDIC International.

Designation	Product Number		Conditions of	
	Without Antibiotics	With Antibiotics	Shipping Temp	Storage Temp
HepaRG® Thawing/Plating/General Purpose Medium Supplement	ADD671C	ADD670C	-20 °C or lower	
HepaRG® Maintenance/Metabolism Medium Supplement	ADD621C	ADD620C	-20 °C or lower	
HepaRG® Induction Medium Supplement	ADD641C	ADD640C	-20 °C or lower	
HepaRG® Serum-free Induction Medium Supplement	ADD651C	ADD650C	-20 °C or lower	

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4. Cells (Immediately place the cryovial(s) in liquid nitrogen upon receipt)

HepaRG® cells	Number of viable cells / vial (× 10^6)	Reference	Conditions of	
			Shipping	Storage
Differentiated HepaRG® cells cryopreserved	≥8	HPR116080**	Dry ice or liquid nitrogen	Liquid nitrogen vapor phase OR -150 °C

**Not Available in Japan

SECTION 2: PROTOCOL

> ***YOUR SAFETY***
> ***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 Media preparation

1.1 BASE MEDIUM

- Add 1 mL of Ala-Gln 200 mM solution to one bottle of 100 mL Williams E Medium
- The Base Medium should be stored at 4 °C.

1.2 HepaRG® THAWING/PLATING/GENERAL PURPOSE MEDIUM 670

- Thaw the HepaRG® Thawing/Plating/General Purpose Medium Supplement (ADD670C) by placing the bottle in a +37 °C water bath until completely thawed.
- Reconstitute the HepaRG® Thawing/Plating/General Purpose Medium 670 by adding the supplement (ADD670C) to 100 mL of Base Medium
- The reconstituted HepaRG® Thawing/Plating/General Purpose Medium 670 is now ready for use. It should be stored at +4 °C for a maximum of 30 days.

1.3 HepaRG® MAINTENANCE/METABOLISM MEDIUM 620

- Thaw the HepaRG® Maintenance/Metabolism Medium Supplement (ADD620C) by placing the bottle in a +37 °C water bath until completely thawed.
- Reconstitute the HepaRG® Maintenance/Metabolism Medium 620 by adding the supplement (ADD620C) to 100 mL of Base Medium.
- The reconstituted HepaRG® Maintenance/ Metabolism Medium 620 is now ready for use. It should be stored at +4 °C for a maximum of 30 days.

1.4 HepaRG® INDUCTION MEDIUM 640

- Thaw the HepaRG® Induction Medium Supplement (ADD640C) by placing the bottle in a +37 °C water bath until completely thawed.
- Reconstitute the HepaRG® Induction Medium by adding the supplement (ADD640C) to 100 mL of Base Medium.
- The reconstituted HepaRG® Induction Medium 640 is now ready for use. It should be stored at +4 °C for a maximum of 30 days.

1.5 HepaRG® SERUM FREE INDUCTION MEDIUM 650

- Use ADD650 in place of ADD640C if you prefer to use Serum-free medium; in such an instance, follow all the steps in 1.3 for use of ADD640C but use ADD650C instead.

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2 Thawing and Seeding HepaRG® cells

2.1 Thawing

- Pre-warm the HepaRG® Thawing/Plating/General Purpose Medium 670 (see 1.1) in the +37 °C water bath.
- Pipette 9 mL (per HepaRG® cryovial to be used) of pre-warmed HepaRG® Thawing/Plating/General Purpose Medium 670 into a sterile 40 mL 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 cap a quarter turn (do not open the cryovial completely) to release the internal pressure, and then close it again.
- 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**. 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.
- Wipe the outside of the cryovial with 70% ethyl alcohol on an 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® Thawing/Plating/General Purpose Medium 670 (resulting in a 1:10 ratio of cell suspension to total volume).
- Rinse out the cryovial once with approximately 1 mL of the HepaRG® Thawing/Plating/General Purpose Medium 670 and return the resulting suspension to the 40 mL tube.
- Centrifuge the differentiated HepaRG® cell suspension **FOR 3 MIN AT 500** × ***g*** at room temperature. Do not follow hepatocyte protocol for centrifugation, the HepaRG® cells are smaller in size and need a longer and faster centrifugation.
- Aspirate the supernatant. To avoid aspiration of cells, leave a small volume of medium on the pellet.
- Resuspend **gently** the differentiated HepaRG® cell pellet with HepaRG® Thawing/Plating/General Purpose Medium 670 in 5 mL for HPR116-TA08, or 7 mL for HPR116-TA12. **Don't try to dissociate the bigger clusters.**

2.2 Cell viability measurement and counting

Cell viability measurement and cell counting are determined by trypan blue (0.05% in D-PBS 1×) exclusion test.

- Transfer 900 µL of trypan blue solution (0.05% in D-PBS 1×) in a 5 mL polystyrene round bottom tube.
- Prepare a cell counting chamber (e.g., Nageotte chamber). To prepare the counting chamber the mirror-like polished surface is carefully cleaned with lens paper. The coverslip is also cleaned. The coverslip is placed over the counting surface before prior to putting on the cell suspension.
- Gently homogenize the cell suspension by manual swirling.
- Dilute 100 µL of the cell suspension in the 900 µL of trypan blue solution at 0.05%.
- Gently homogenize the obtained cell suspension.
- Introduce with a pipette around 100 µL of cell suspension between mirror-like polished surface and coverslip. The area under the coverslip fills by capillary action. Enough liquid should be introduced so that the mirrored surface is just covered.
- Observe under microscope.

  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.

  Careful: Thawed differentiated HepaRG® cells can form clusters. It's necessary to count all the cells including those forming the clusters.
- Determine the average number of viable cells and dead cells per row.

- Determine percentage of cell viability.

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

- Calculate the cell concentration in million cells/mL.
  Sample calculation with a Nageotte chamber:

  Number of viable cells per row × **10** (dilution factor in trypan blue) × **800** (parameter relating to the Nageotte cell)= M cell/mL

- Calculate the total viable cell number:

  Cell concentration in million cells/mL × Total volume of cell suspension = total number of cells

3 Use of differentiated HepaRG® cells

3.1 METABOLISM studies: use of HepaRG® IN SUSPENSION

- After thawing and counting of differentiated HepaRG® cells (Section 2), cells can be used for the metabolism studies in suspension according to your standard protocol with human hepatocytes.
- Incubate the cells with the test substrates according to your protocol for metabolism studies.

SUSPENSION	**Day 1**	- Thaw the cells in HepaRG® Thawing/Plating/General Purpose Medium 670 - Incubate the cells with the test substrates according to your protocol

3.2 METABOLISM studies: use of HepaRG® IN MONOLAYER

3.2.1 Cell seeding

- After the thawing and the counting of differentiated HepaRG® cells (Section 2), and using the Thawing/Plating/General Purpose Medium 670, seed the differentiated HepaRG® cells into flat-bottom multiwell plate(s)) or flask(s) according to the table below:

Cryopreserved differentiated HepaRG® cells			
Cell culture support	**Number of viable cells per well (× 10^6)**	**Volume per well/flask (mL)**	**Cell concentration (× 10^6/mL)**
25 cm² flask	5.2	5	1.04
6 well plate	2	2	1
12 well plate	0.8	1	0.8
24 well plate	0.48	0.5	0.96
48 well plate	0.16	0.2	0.8
96 well plate*	0.072	0.1	0.72

* If 96 well plate(s) are seeded partially, fill the wells surrounding those containing the cells with sterile water.

- Except for the 96 well-plates, gently agitate the supports in a back-and-forth and side-to-side manner and visually control the homogeneity of the cell distribution.
- Place the plate(s) or flask(s) in the incubator at +37 °C, 5% CO_2, and saturating humidity.

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3.2.2 Cell maintenance for metabolism studies

You have two options:
Either use the cells immediately after thawing, or following at least 3 days of culture. HepaRG® keep 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 8.

> **<u>At day 1, 4 hours after plating</u>**

- Four hours after plating, observe cell morphology under phase-contrast microscope, and when possible, take photomicrographs.
- Cells can be used for the metabolism studies according to your standard protocol with human hepatocytes.
- Incubate the cells with the test substrates according to your protocol for metabolism studies.

MONOLAYER Day 1	**4 hours after plating**	- Thaw and seed the cells using HepaRG® Thawing/Plating/General Purpose Medium 670 - Four hours after plating, incubate the cells with the test substrates according to your protocol

> **<u>Day 5-Day 8</u>**

- One day after thawing, observe cell morphology under phase-contrast microscope, and when possible, take photomicrographs.
- Change from the HepaRG® Thawing/Plating/General Purpose Medium 670 to the HepaRG® Maintenance/Metabolism Medium 620.

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

- Pre-warm the HepaRG® Maintenance/Metabolism Medium 620 in a sterile container (12 mL/24 or 12 well plate, 9.6 mL/48 or 96 well plate, plus a little extra) at room temperature.
- Transfer the HepaRG® Maintenance/Metabolism Medium 620 pre-warmed into a 92 × 17 mm Petri dish or similar flat-bottom container suitable for use with multichannel pipette.
- Remove the lid from the multiwell plate.
- Remove the existing medium from the wells.
- Gently add the pre-warmed HepaRG® Maintenance/Metabolism Medium 620 to the sides of each well with a multichannel pipette (for volume per well, see upper table in paragraph 3.2.1). Do not add the medium directly onto the cells.
- Control visually the medium level in the wells.
- Put the lid back on the multiwell plate and place the plate(s) back in the +37 °C incubator.

For 6 well plate(s):

- Pre-warm the HepaRG® Maintenance/Metabolism Medium 620 in a sterile container (12 mL/6 well plate) at room temperature.
- Remove the lid from the multiwell plate.
- Remove the existing medium from the wells.
- Gently add the pre-warmed HepaRG® Maintenance/Metabolism Medium 620 to the sides of each well with a pipette (2 mL per well). Do not add the medium directly onto the cells.
- Control visually the medium level in the wells.
- Put the lid back on the multiwell plate and place the plate(s) back in the +37 °C incubator.

For 25 cm² flask(s):

- Pre-warm the HepaRG® Maintenance/Metabolism Medium 620 at room temperature.
- Remove the cap from the flask(s).
- Aspirate the existing medium from the flask.
- Transfer 5 mL of pre-warmed HepaRG® Maintenance/Metabolism Medium 620 into the 25 cm² flask. Take care not to pipette down the medium directly on the cells.
- Replace the cap on the flask and place the flask(s) back in the +37 °C incubator.

- Maintain the HepaRG® cells in HepaRG® Maintenance/Metabolism Medium 620 and use the cells:

> **At Day 5**

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

MONOLAYER Use at Day 5 96 hrs	**Day 1**	**Thursday**	Thaw and seed the cells using HepaRG® Thawing/Plating/General Purpose Medium 670
	Day 2 24 hrs	**Friday**	Remove HepaRG® Thawing/Plating/General Purpose Medium 670, and replace with the HepaRG® Maintenance/Metabolism Medium 620
	Day 5 96 hrs	**Monday**	Incubate the cells in monolayer with the test substrates according to your protocol

> **At Day 8**

For optimal activity levels, Maintenance/Metabolism Medium 620 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.

MONOLAYER Use at Day 8 168 hrs	**Day 1**	**Thursday**	Thaw and seed the cells using HepaRG® Thawing/Plating/General Purpose Medium 670
	Day 2 24 hrs	**Friday**	Remove HepaRG® Thawing/Plating/General Purpose Medium 670, and replace with HepaRG® Maintenance/Metabolism Medium 620
	Day 5 96 hrs	**Monday**	Renew the HepaRG® Maintenance/Metabolism Medium 620
	Day 7 144 hrs	**Wednesday**	Renew the HepaRG® Maintenance/Metabolism Medium 620
	Day 8 168 hrs	**Thursday**	Incubate the cells in monolayer 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 with human hepatocytes. They can also be kept in HepaRG® Maintenance/Metabolism Medium 620 for 1 additional week, provided that renewal of the HepaRG® Maintenance/Metabolism Medium 620 is performed every 2-3 days.**

3.3 Induction studies: use of HepaRG® in monolayer

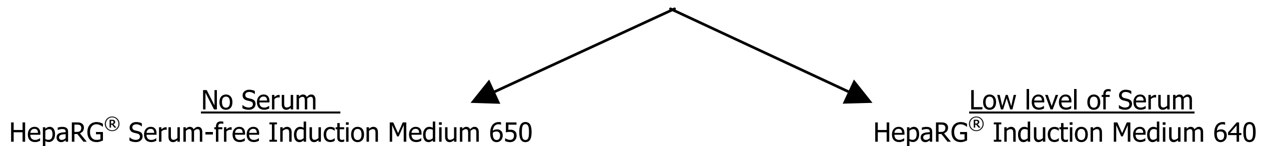
3.3.1 Cell seeding

See 3.2.1

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.
- At day 4, after 72 hours of culture, observe cell morphology under phase-contrast microscope, and when possible, take photomicrographs.

- Cells can be used for induction studies: choose between two media with:



<u>No Serum</u>
HepaRG® Serum-free Induction Medium 650

<u>Low level of Serum</u>
HepaRG® Induction Medium 640

- Change from the HepaRG® Thawing/Plating/General Purpose Medium 670 to either the HepaRG® Induction Medium 640 or HepaRG® Serum-free Induction Medium 650 with the test articles.
- Incubate the cells with the test articles for 48 hours.
- Renew the medium with the test articles daily and always with the medium chosen at the beginning of the study (either HepaRG® Induction Medium 640 or HepaRG® Serum-free Induction Medium 650).

> ***Note*:** **Maximal fold induction of metabolic activity <u>may</u> be achieved with 72 hours treatment time, but vendor's 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 hours treatment time is applied in most cases, but 48 hours incubation is also retained by some users.**

3.3.3 Suggested timeline for induction studies

Day 1	**Friday morning**	Thaw and seed the cells using HepaRG® Thawing/Plating/General Purpose Medium 670
Day 1 **6 hrs**	**Friday end of afternoon (6 hrs after plating)**	Observe cell morphology under phase contrast microscope, and when possible, take photomicrographs
Day 4 **72 hours**	**Monday morning**	Remove the HepaRG® Thawing/Plating/General Purpose Medium 670, and replace with the HepaRG® Induction Medium 640 or HepaRG® Serum-free Induction Medium 650 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 5 **96 hours**	**Tuesday morning**	Renew the HepaRG® Induction Medium 640 or HepaRG® Serum-free Induction Medium 650 with the test articles
Day 6 **120 hours**	**Wednesday morning**	End of the incubation with the test articles Incubate the cells with the test substrates

3.4 UPTAKE AND TRANSPORT studies: use of HepaRG® IN SUSPENSION

- After thawing and counting of differentiated HepaRG® cells (Section 2), cells can be used for uptake and transport studies in suspension according to your standard protocol with human hepatocytes.
- Incubate the cells with the test substrates according to your protocol for uptake and transport studies.

SUSPENSION	**Day 1**	- Thaw the cells in HepaRG® Thawing/Plating/General Purpose medium 670 - Incubate the cells with the test substrates according to your protocol

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3.5 TOXICITY studies: use of HepaRG® IN MONOLAYER

3.5.1 Cell seeding

See 3.2.1

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.

- Renew the HepaRG® Thawing/Plating/General Purpose Medium 670 (for method, see 3.2.2).
- Maintain the HepaRG® cells in HepaRG® Thawing/Plating/General Purpose Medium 670 until the use of cells at day 8.

- Renew the HepaRG® Thawing/Plating/General Purpose Medium 670, and incubate the cells in monolayer with the test articles according to your protocol.

3.5.3 Suggested timeline for toxicity studies

Day 1	**Thursday**	Thaw and seed the cells using HepaRG® Thawing/Plating/General Purpose Medium 670
Day 2 **24 hours**	**Friday**	Renew the HepaRG® Thawing/Plating/General Purpose Medium 670
Day 5 **96 hours**	**Monday**	Renew the HepaRG® Thawing/Plating/General Purpose Medium 670
Day 7 **144 hours**	**Wednesday**	Renew the HepaRG® Thawing/Plating/General Purpose Medium 670
Day 8 **168 hours**	**Thursday**	Remove the Thawing/Plating/General Purpose Medium 670 and incubate the cells in monolayer with the test articles according to your protocol

See the next page for photomicrographs of HepaRG® cells in culture at different time points.

SECTION 3: CELL MORPHOLOGY

- After 24 hours of culture, hepatocyte-like cells appear in small, differentiated colonies, individualized (see Figure 1).
- After 72-96 hours of culture, a restructuring of cell monolayer can be observed with a hepatocyte-like cells' organization in clusters (see Figure 2).
- 120-144 hours after plating, hepatocyte-like cells are organized in well-delineated trabeculae with many bright canaliculi-like structures (see Figure 3).

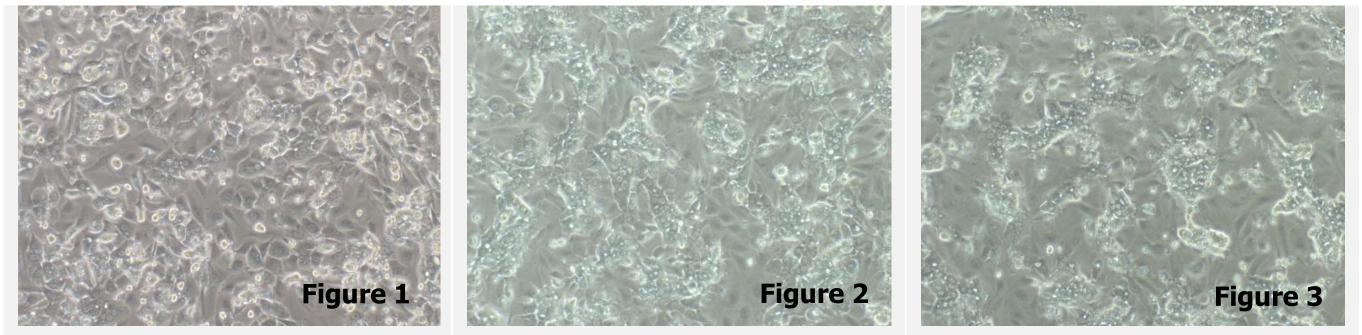
Figure 1 Figure 2 Figure 3