



DIFFERENTIATED HepaRG[®]-NS CRYOPRESERVED

Description and user guide For thawing, culture and use

Catalog number: HPR116NS

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.

HPR116NS is a new format of the HPR116 with a freezing process that allows direct thawing and seeding of cryopreserved differentiated cells without the need for post-thaw washing, centrifugation and counting steps.

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

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For more information and all publications on HepaRG[®], visit

www.HepaRG.com

SECTION 1: MATERIALS, MEDIA AND CELLS

1. Materials

- Water bath at +37°C
- Laminar flow hood
- Pipet-aid, pipettes and micropipettes
- Multichannel pipettes
- 70% alcohol prep/wipe/swab
- 40 mL Polystyrene round-bottom tubes and 92 x 17 mm petri dishes or similar containers
- Incubator at +37°C, 5% CO₂ 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[®], HPR116NS.

Designation	Reference	Conditions of	
		Shipping	Storage
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

* BIOPREDIC International proprietary coating process to ensure proper seeding and culture of the HPR116NS
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
Williams E Medium, no phenol red	Invitrogen™	A12176-01*	+4°C
GlutaMAX™ I, 200 mM	Invitrogen™	35050*	-20°C

BIOPREDIC International recommends Williams E Medium and GlutaMAX™-I from Invitrogen™ only.

*References in North America

The following supplements are available from BIOPREDIC International.

Designation	Reference	Conditions of	
		Shipping	Storage
HepaRG [®] -NS Thawing and Plating Medium Supplement with corticoids	ADD411	-20°C or lower	
HepaRG [®] -NS Maintenance/Metabolism Medium Supplement with corticoids	ADD421	-20°C or lower	
HepaRG [®] -NS Pre-induction and Tox Medium Supplement with corticoids	ADD431	-20°C or lower	
HepaRG [®] -NS Serum-free Induction Medium Supplement with corticoids	ADD451	-20°C or lower	

4. Cells

HPR116NS \geq 8M viable cells / 2 mL vial, shipped on dry ice or in LN dewar. If on dry ice, ensure shipment time did not exceed 48 hours. Immediately place the cryovial(s) in liquid nitrogen upon receipt. Keep the certificate of analysis handy for calculating the total suspension volume, or record the total population of viable cells according to the certificate of analysis information for use in section 2, 2.2.

SECTION 2: PROTOCOL FOR THE THAWING, SEEDING AND MAINTENANCE OF DIFFERENTIATED HEPARG[®]-NS CRYOPRESERVED

YOUR SAFETY

OBSERVE UNIVERSAL PRECAUTIONS WHEN HANDLING HepaRG[®]-NS 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 preparation

Combine 99 mL of Invitrogen Williams E medium # A12176-01 with 1 mL of Invitrogen GlutaMAX™ I # 35050 in a sterile container.

1.2 Working Medium Preparation

- Thaw the HepaRG[®]-NS Supplement by placing the bottle in a +37°C water bath until completely thawed.
- Prepare working HepaRG[®]-NS Medium by adding the entire content of the bottle of HepaRG[®]-NS Supplement to 100 mL of Base Medium.
- Working HepaRG[®]-NS Medium should be stored at +4°C for a maximum of one month. See Section 2, 4 for detailed instructions about medium change.

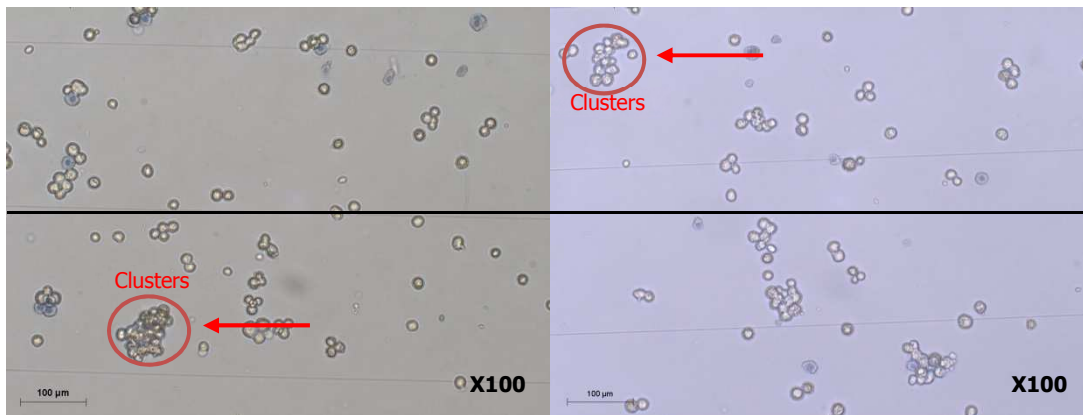
2 Thawing and Seeding HepaRG[®] cells

2.1 Thawing and suspending cells

1. Pre-warm working HepaRG[®]-NS Thawing/Plating Medium 411 in a +37°C water bath.
2. For the thawing of one vial, pipet 7.5 mL of pre-warmed, working HepaRG[®]-NS Thawing/Plating Medium 411 into a sterile 40 mL polystyrene round-bottom tube or similar container.
3. Remove the cryovial from the liquid nitrogen storage.

Note: **Steps 4, 5, and 6 need to be accomplished within four minutes to avoid risk of any room temperature thawing which will affect cell viability.**

4. Under the laminar flow hood, briefly twist the cryovial cap a quarter to release internal pressure, and then close it again a quarter turn.
5. Quickly transfer the cryovial into the +37°C water bath. Do not submerge the vial completely, do not allow water to penetrate into the cap. While holding the tip of the cryovial, agitate the vial in a back and forth manner for 90 seconds. Small ice crystals should remain when removed from the water bath.
6. Under the laminar flow hood, wipe the outside of the cryovial with the 70% ethyl alcohol. Then aseptically transfer with a pipette the "semi"-thawed HepaRG[®]-NS cell suspension into the 40 mL tube containing the pre-warmed working HepaRG[®]-NS Thawing/Plating Medium.
7. Pipet approximately 1 mL of the cell suspension from 2.1.6. above into the cryovial, swirl it, and return the resulting suspension to the 40 mL tube, to ensure you have recovered all cells.
8. Reduce cell clusters by using a pipette by gently drawing the suspension into the pipette and then pipeting back out. The cell suspension should be homogeneous to allow cells to plate evenly, but, under a microscope, it is normal to have some clusters, as shown below.



2.2 Seeding the thawed HepaRG®-NS

Note: HPR116NS is a new format that allows direct thawing and seeding of cryopreserved differentiated cells without the need for post-thaw washing, centrifugation and counting steps.

But if a counting is needed, follow instruction in step 3.

2.2.1 Calculating the total volume of suspension/vial of cells

The final volume of suspended cells needed for different well plate formats is provided on each lot's Certificate Of Analysis (Sec 3, Conditions of Cell Seeding) based on the total population of viable cells/vial for that specific lot. Once the cells are thawed properly the initial volume will be 8 mL, so the total volume shown includes that; in other words, if the total volume shown on the COA is 10.4 mL, then 2.4 mL of working HepaRG®-NS Thawing/Plating Medium should be added to the tube with the thawed cells.

For general calculation of vials and media needed, following are the volumes of suspension and cells per well or per plate for different multiwell plate configurations when the viable cell population in a vial is 8M cells:

Well plate	Total volume of Media with Suspended Cells (mL)		Required number of cells (10 ⁶)	
	Per Well	Per Plate	Per well	Per Plate
6 well plate	2	12	2	12
12 well plate	1	12	0.8	9.6
24 well plate	0.5	12	0.48	11.5
48 well plate	0.2	9.6	0.16	7.7
96 well plate	0.1	9.6	0.072	6.9

2.2.2 Seeding the plates

According to the cell culture support used, dispense in each well the volume of suspended cells indicated in the table above, with a micropipette or a multichannel pipette.

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.

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

Place the plate(s) in the incubator at +37°C, 5% CO₂ and saturating humidity.

3 Cell viability and counting

Optional and not recommended

Note: Steps 2.1 and 3 need to be accomplished within 30 minutes to avoid damaging the cells.

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

1. Transfer 900 μL of trypan blue solution (0.05% in D-PBS 1X) in a 5 mL polystyrene round bottom tube.
2. 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.
3. Gently homogenize the cell suspension by manual swirling.
4. Dilute 100 μL of the cell suspension in the 900 μL of trypan blue solution at 0.05%.
5. Gently homogenize the obtained cell suspension.
6. 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.
7. Proceed to observation under microscope.
 - o 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 very different from one row to another, count one or two more rows.
 - o **Careful:** Thawed differentiated HepaRG[®]-NS cells can form clusters. It's necessary to count all the cells including those forming the clusters.
8. Determine the average number of viable cells and dead cells per row.
9. Determine percentage of cell viability.

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

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

$$\text{Number of viable cells per row} \times 10 \text{ (dilution factor in trypan blue)} \times 800 \text{ (parameter relating to the Nageotte cell)} = \text{M cell / mL}$$

11. Calculate the total viable cell number:

$$\text{Cell concentration in million cells / mL} \times \text{Total volume of cell suspension} = \text{total number of cells}$$

4 Medium change

Suggestions about removing/adding culture medium from well plates with monolayer cells.

4.1 Removing culture medium

- o Slightly tilt the plate to reach the medium in the bottom of each well. You can use a vacuum aspiration system or manual pipetor, but leave a small volume of medium in the well, doing so helps to avoid touching the monolayer.
- o Take care to avoid touching the monolayer with pipet tips.

4.2 Replenishing with new culture medium

- o Pipet new medium down the walls of each well, let the fluid first touch the plastic and then collect on the monolayer.
- o Using a multichannel pipettes is recommended, and avoid high velocities in pipeting the medium into the wells.

5 Use of differentiated HepaRG®-NS cells

5.1 METABOLISM Studies: Use of HepaRG® in suspension

- After thawing of differentiated HepaRG® cells (Section 2, 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	<ul style="list-style-type: none"> - Thaw the cells using HepaRG®-NS Thawing/Plating Medium 411 - Incubate the cells with the test substrates according to your protocol
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5.2 METABOLISM studies: use of HepaRG® in monolayer

5.2.1 Cell seeding
See section 2, 2.2

5.2.2 Cell maintenance for metabolism studies

You have two options:

Either use the cells rapidly 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 7.

➤ **At day 1, 4 hours after plating**

- Four hours after plating, observe cell morphology under phase-contrast microscope, and when possible, take photomicrographs, for your own records and also for the best assistance from your vendor's experts. It is difficult for the HepaRG® experts to diagnose issues and propose solutions without pictures.
- 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	<ul style="list-style-type: none"> - Thaw and seed the cells using HepaRG®-NS Thawing/Plating Medium 411 - Four hours after plating, incubate the cells with the test substrates according to your protocol
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➤ **Day 5-Day 8**

- One day after thawing, observe cell morphology under phase-contrast microscope, and when possible, take photomicrographs.
- Change from the HepaRG®-NS Thawing/Plating Medium 411 to the HepaRG® Maintenance/Metabolism Medium 421.
- Maintain the HepaRG® cells in HepaRG® Maintenance/Metabolism Medium 421 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 [®] -NS Thawing/Plating Medium 411
	Day 2 24 hrs	Friday	Remove HepaRG [®] -NS Thawing/Plating Medium 411, and replace with the HepaRG [®] Maintenance/Metabolism Medium 421
	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 421 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 [®] -NS Thawing/Plating Medium 411
	Day 2 24 hrs	Friday	Remove HepaRG [®] -NS Thawing/Medium 411, and replace with the HepaRG [®] Maintenance/Metabolism Medium 421
	Day 5 96 hrs	Monday	Renew the HepaRG [®] Maintenance/Metabolism Medium 421
	Day 7 144 hrs	Wednesday	Renew the HepaRG [®] Maintenance/Metabolism Medium 421
	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[®]-NS Maintenance/Metabolism Medium 421 for 1 additional week, provided that renewal of the HepaRG[®]-NS Maintenance/Metabolism Medium 421 is performed every 2-3 days.

5.3 INDUCTION Studies5.3.1 Cell seeding

See section 2, 2.2

5.3.2 Culture and maintenance for induction studies

- Six hours after plating, observe cell morphology under phase contrast microscope, and when possible, take photomicrographs.
- Remove HepaRG[®]-NS Thawing/Plating Medium 411, and replace with the Pre-induction/Tox Medium 431. Volumes are specified in the tables in section 2, 2.2.
- At day 4, after 72 hours of culture, observe cell morphology under phase-contrast microscope, and when possible, take photomicrographs.

- Change from the HepaRG[®]-NS Pre-induction/Tox Medium 431 to HepaRG[®]-NS Serum-free Induction Medium 451 with the test articles.
- Incubate the cells with the test articles for 48h.
- Renew the medium with the test articles daily with the HepaRG[®]-NS Serum-free Induction Medium 451.

Note: Maximal fold induction of metabolic activity may 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.

5.3.3 Suggested timeline for induction studies

Day 1	Friday morning	Thaw and seed the cells using HepaRG [®] -NS Thawing/Plating Medium 411.
Day 1 6 hrs	Friday end of afternoon (6 h after plating)	Remove HepaRG [®] -NS Thawing/Plating Medium 411, and replace with the Pre-induction/Tox Medium 431.
Day 4 72 hours	Monday morning	Remove the HepaRG [®] -NS Pre-induction/Tox Medium 431, and replace with the HepaRG [®] -NS Serum-free Induction Medium 451. 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 [®] -NS Serum-free Induction Medium 451 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.

5.4 TOXICITY Studies

5.4.1 Cell seeding

See section 2, 2.2

5.4.2 Culture and maintenance for toxicity studies

- One day after thawing, observe cell morphology under phase-contrast microscope, and when possible, take photomicrographs.
- Remove HepaRG[®]-NS Thawing/Plating Medium 411, and replace with the Pre-induction/Tox Medium 431.
- Maintain the cells in HepaRG[®]-NS Pre-induction/Tox Medium 431 until the use of cells, at least 7 days, with a medium renewal every 2-3 days.
- Renew the HepaRG[®]-NS Pre-induction/Tox Medium 431, and incubate the cells in monolayer with the test articles according to your protocol.

5.4.3 Suggested timeline for toxicity studies at day 7

Day 1	Thursday	Thaw and seed the cells using HepaRG [®] -NS Thawing/Plating Medium 411.
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Day 2 24 hours	Friday	Remove HepaRG [®] -NS Thawing/Plating Medium 411, and replace with the Pre-induction/Tox Medium 431.
Day 5 96 hours	Monday	Renew the HepaRG [®] -NS Pre-induction/Tox Medium 431.
Day 7 144 hours	Wednesday	Renew the HepaRG [®] -NS Pre-induction/Tox Medium 431.
Day 8 168 hours	Thursday	Remove the HepaRG [®] -NS Pre-induction/Tox Medium 431 and incubate the cells in monolayer with the test articles according to your protocol

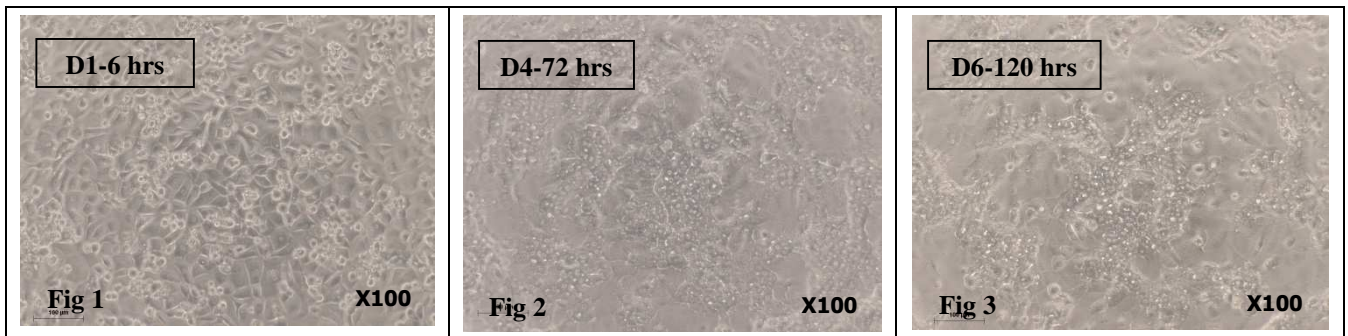
5.5 UPTAKE AND TRANSPORT studies: use of HepaRG[®] IN SUSPENSION

- After thawing of differentiated HepaRG[®] cells (Section 2, 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	<ul style="list-style-type: none"> - Thaw the cells using HepaRG[®]-NS Thawing/Plating Medium 411 - Incubate the cells with the test substrates according to your protocol
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SECTION 3: CELL MORPHOLOGY

- 6 hours after plating, cells attach and spread to form a monolayer close to the one observed with human hepatocytes (fig 1).
- After 72-96 hours of culture, a restructuring of cell monolayer can be observed with a hepatocyte-like cells' organization in clusters (fig 2).
- 120 hours after plating, hepatocyte-like cells are organized in well-delineated trabeculae with many bright canalliculi-like structures (fig 3).



Note: Photomicrographs taken on 96 well plates to be closer with reality, explaining a slight optical distortion.