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Differentiated NoSpin HepaRG™ Cryopreserved Cells

Instructions for Use

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I. Introduction

HepaRG™ cells have unique properties. They maintain significant levels of hepatic cell functions, are CYP450 inducible and support the complete replicative cycle of Hepatitis B Virus (HBV). NoSpin HepaRG™ (NSHPRG) cells are a new HepaRG™ cell format prepared with a freezing process that allows direct thawing and seeding of cryopreserved differentiated cells without the need for post-thaw washing, centrifugation and counting. Lonza HepaRG™ cells are offered in cryopreserved vials containing ≥ 8M viable cells per vial.

II. Required Materials

1. HepaRG™ cells (Lonza catalog number NSHPRG)
2. Basal medium (Lonza catalog number MH100-1)
3. Basal medium supplement (Lonza catalog number MH100-2)
4. Media additives (purchased separately), see Table 1
5. Rat tail type I collagen coated plates. This is recommended for optimal HepaRG™ cell attachment.
6. Optional: antibiotics to supplement HepaRG™ working medium (i.e. penicillin/streptomycin or gentamicin/amphotericin)

III. Unpacking and Storage Instructions

1. Check all containers for leakage or breakage.

2. Remove the cryovials containing the cells from the dry ice packaging and immediately store them in liquid nitrogen. Alternatively, thaw and use the cells immediately. If no dry ice remains, please contact Customer Service.
3. Store basal medium (MH100-1) and basal medium supplement (MH100-2) at 4ºC upon arrival. Store frozen additives at -20°C in a freezer that is not self-defrosting. If thawed upon arrival, the additives can be stored at 2°- 8°C and added to the basal medium within 72 hours of receipt. After NoSpin HepaRG™ additives are added to basal medium, use within one month. Do not re-freeze.

NOTE: Using media or reagents other than what is recommended will void the cell warranty. Please contact Scientific Support if you need help selecting media and/or reagents.

IV. Preparation of Medium

1. *Complete Basal Medium*
    a. Add the entire contents of the tube of NoSpin HepaRG™ Basal Medium Supplement (MH100-2) to the 100 mL bottle of NoSpin HepaRG™ Basal Medium (MH100-1). This will be referred to as ***complete basal medium***. The total volume of the complete basal medium will be 101 mL.
    b. Optional: Add desired antibiotics to appropriate concentration based on application or need.
    c. Store NoSpin HepaRG™ complete basal medium at 4°C for a maximum of one month.

2. *Working Medium*
    a. Depending on the application, prepare your working media by adding the entire contents of the appropriate additive to the NoSpin HepaRG™ complete basal medium, according to Table 1. Store at 4°C. Each working medium will expire one month from the date the complete basal medium was supplemented.

Table 1. Media Additives

Application(s)	Thawing, Plating, Transporter Evaluation	Plated Metabolism, General Cell Culture Maintenance	P450 Pre-induction, Toxicity Testing	P450 Induction
Additive	NoSpin HepaRG™ Thawing and Plating Additive	NoSpin HepaRG™ Maintenance/Metabolism Additive	NoSpin HepaRG™ Pre-induction and Tox Additive	NoSpin HepaRG™Serum-free Induction Additive
Catalog Number	MHTAP	MHMET	MHPIT	MHIND
Additive Volume	11.8 mL	14 mL	12.5 mL	0.6 mL
Complete Basal Medium Volume	101 mL	101 mL	101 mL	101 mL
Working Medium Volume	112.8 mL	115 mL	113.5 mL	101.6 mL

V. Thawing of Cells / Initiation of Culture Process

Working Media Needed
MH100 complete basal medium + MHTAP Supplement

1. Warm the working NoSpin HepaRG™ Thawing and Plating Medium (e.g. complete basal medium plus thawing and plating additive) in a 37°C water bath.
2. Pipet 7.5 mL of pre-warmed working medium into a sterile 40 mL polystyrene round-bottom tube (or similar). Maintain the tube containing the medium at 37°C.
3. Remove the cryopreserved vial of cells from the liquid nitrogen storage.

NOTE: Accomplish steps 4, 5, and 6 within 4 minutes to avoid room temperature thawing which impacts cell viability.

4. Wipe cryovial with ethanol or isopropanol before opening. In a sterile field, briefly twist the cap a quarter turn to release pressure, and then retighten.
5. Quickly transfer the cryovial into the 37°C water bath. Do not submerge the vial completely and do not allow water to penetrate into the cap. Agitate the vial back and forth for 90 seconds. Small ice crystals should remain when the vial is removed from the water bath.
6. Wipe the outside of the cryovial with ethanol or isopropyl alcohol. With a pipette, carefully mix the 0.5 mL of cell suspension and then aseptically transfer it into the 40 mL tube containing the pre-warmed working NoSpin HepaRG™ Thawing and Plating Medium.
7. To recover all cells, pipet approximately 1 mL of the cell suspension from the 40 mL tube back into the cryovial, swirl it, and then add it back to the 40 mL tube. The total cell suspension volume will be 8 mL.
8. Reduce cell clusters by gently pipetting the suspension a few times. Some remaining clusters are normal.
9. The final volume of the suspended cells needed for different plate formats is provided on the Certificate of Analysis (COA) for each lot and is based on the total population of viable cells for that specific lot. For example, if the total volume shown on the COA for the plating format is 10.4 mL, an additional 2.4 mL of the working NoSpin HepaRG™Thawing and Plating Medium should be added to the 8 mL of cell suspension prepared in step 7. The suspension should be mixed gently. General plating volume and cell number information is provided in table 2.

NOTE: If using cells for suspension applications, skip to section VI .

10. Pipette the appropriate volume of suspended cells noted for your plating format as indicated in Table 2.
11. For all plating formats (except 96 well plates), gently move the culture vessel back-and-forth and side-to-side to ensure homogeneous cell distribution. If using 96 well plate(s) and they are partially seeded, fill the wells surrounding those containing the cells with sterile water.
12. Incubate plate(s) at 37°C, 5% CO_2 and 90% ± 2% humidity.

Table 2. Volumes of suspension and cells per well or per plate for multiwell plate configurations

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

NOTE: In this example, the viable cell population is 8M cells

VI. Application Guidelines

1. *Uptake and Transport Studies: Use of HepaRG™ in Suspension*
    a. After thawing of differentiated HepaRG™ cells (Section V), cells can be used for uptake and transport studies in suspension according to your standard protocol for human hepatocytes.
    b. Incubate the cells with the test substrates according to the uptake and transport studies protocol.

2. *Metabolism Studies: Use of HepaRG™ Cells in Suspension*

Working Media Needed
MH100 complete basal medium + MHTAP Supplement

    a. After thawing the differentiated HepaRG™ cells (Section V), the cells can be used for metabolism studies in suspension according to a standard protocol for human hepatocytes.
    b. Incubate the cells with the test substrates according to your application.

3. *Metabolism Studies: Use of HepaRG™ in a Monolayer*

NOTE: There are two options when using cells for metabolism studies, the cells may be used immediately after plating, or following at least 3 days of culture. HepaRG™ cells retain a high level of CYP activities during the first 24 hours following thawing and plating, then these activities decrease while the cells reconstitute the monolayer. Activities return during the 4th day in culture, peaking at day 8.

Working Media Needed
MH100 complete basal medium + MHTAP Supplement
MH100 complete basal medium + MHMET Supplement

    a. Metabolism Studies Immediately After Plating
        1. Cell seeding – See Section V
        2. Four hours after plating, observe cell morphology under a phase-contrast microscope. When possible, take photomicrographs for laboratory records. Note that it is difficult for the HepaRG™ experts to diagnose issues and propose solutions without images.
        3. Incubate the cells with the test substrates according to the metabolism study protocol.

    b. Metabolism Studies After Establishing Monolayer (Days 5-8)
        1. Cell seeding – See Section V
        2. On day 2, after 24 hours in culture
            i. Observe the cell morphology under a phase-contrast microscope, and take photomicrographs when possible. Note that it is difficult for the HepaRG™ experts to diagnose issues and propose solutions without pictures
            ii. Replace the NoSpin HepaRG™ Thawing and Plating Medium with HepaRG™ Maintenance / Metabolism Medium.
            iii. **Option 1: Day 5 treatment**. On day 5, incubate the cells with the test substrates according to the metabolism study protocol.

Table 3. Suggested schedule for metabolism on Day 5.

Monolayer	Day 1	Thaw and seed the cells using NoSpin HepaRG™ Thawing and Plating Medium
Use at Day 5 **96 hrs**	Day 2 24 hrs	Remove NoSpin HepaRG™ Thawing and Plating Medium, and replace with the HepaRG™ Maintenance / Metabolism Medium
	Day 5 96 hrs	Incubate the cells in monolayer with the test substrates according to your protocol

NOTE: After 5 days in culture, a cell monolayer can be observed with a hepatocyte-like cell organization in clusters and metabolic activities are slightly lower than activities detected for fresh cells.

iv. **Option 2: Day 8 treatment**

1. On day 5 and on day 7, replace the HepaRG™ Maintenance and Metabolism Medium and continue the culture incubation period. This medium change is required to obtain optimal activity levels for cells.
2. On day 8, incubate the cells with the test substrates according to the metabolism study protocol.

Table 4. Suggested schedule for monolayer metabolism on Day 8

Monolayer	**Day 1**	Thaw and seed the cells using NoSpin HepaRG™ Thawing and Plating Medium
Use at Day 8 **168 hrs**	**Day 2** **24 hrs**	Remove NoSpin HepaRG™ Thawing and Plating Medium, and replace with the HepaRG™ Maintenance / Metabolism Medium
	Day 5 **96 hrs**	Renew the HepaRG™ Maintenance / Metabolism Medium
	Day 7 **144 hrs**	Renew the HepaRG™ Maintenance / Metabolism Medium
	Day 8 **168 hrs**	Incubate the cells in monolayer with the test substrates according to your protocol. At day 8 the cells are organized in well-delineated trabeculae with many bright canaliculi-like structures and basal metabolic activities are similar to fresh cells.

NOTE: HepaRG™ cells may be kept in NoSpin HepaRG™ Maintenance / Metabolism Medium for 1 additional week, provided that the NoSpin HepaRG™ Maintenance / Metabolism Medium is replaced every 2-3 days.

4. Induction Studies

Working Media Needed
MH100 complete basal medium + MHTAP Supplement
MH100 complete basal medium + MHPIT Supplement
MH100 complete basal medium + MHIND Supplement

a. Cell seeding – See Section V
b. Six hours after plating, observe cell morphology under a phase contrast microscope, and take photomicrographs when possible.
c. Remove the NoSpin HepaRG™ Thawing and Plating Medium and replace it with the Pre-induction and Tox Medium.
d. Renew the medium with the test compounds daily with the NoSpin HepaRG™ Pre-induction and Tox Medium.
e. On day 4, after 72 hours of culture, observe cell morphology under a phase-contrast microscope and take photomicrographs when possible. Remove the NoSpin HepaRG™ Pre-induction and Tox Medium and replace it with the NoSpin HepaRG™ Serum-free Induction Medium with the test compounds.
f. On day 5, renew the medium with the test articles with the HepaRG™-NS Serum-free Induction Medium.
g. On day 6, the incubation period with the test compounds is complete. Cells should be incubated with the appropriate enzyme substrates for analysis.

NOTE: Maximal fold induction of metabolic activity may be achieved with 72 hours treatment time, but vendor data indicates 48 hours of treatment is sufficient to demonstrate significant induction of CYP1A2, CYP2B6, and CYP3A4 metabolic activity using prototypical inducers.

Table 5. Suggested schedule for Induction Studies

Day 1	Thaw and seed the cells using NoSpin HepaRG™ Thawing and Plating Medium.
Day 1 **6 hrs**	Remove NoSpin HepaRG™ Thawing and Plating Medium, and replace with the Pre-induction and Tox Medium.
Day 4 **72 hours**	Remove the NoSpin HepaRG™ Pre-induction and Tox Medium, and replace with the NoSpin HepaRG™ Serum-free Induction Medium. Incubate the cells in monolayer with the test compounds according to your study design.
Day 5 **96 hours**	Replace the NoSpin HepaRG™ Serum-free Induction Medium with the test compounds.
Day 6 **120 hours**	End of the incubation with the test compounds. Incubate the cells with the appropriate enzyme substrates.

5. Toxicity Studies

Working Media Needed
MH100 complete basal medium + MHTAP Supplement
MH100 complete basal medium + MHPIT Supplement

a. Cell seeding – See Section V
b. On day 2 (24 hours after plating), observe the cell morphology under a phase contrast microscope, and take photomicrographs when possible. Note that it is difficult for the HepaRG™ experts to diagnose issues and propose solutions without images. Remove NoSpin HepaRG™ Thawing and Plating Medium, and replace with the Pre-induction and Tox Medium.

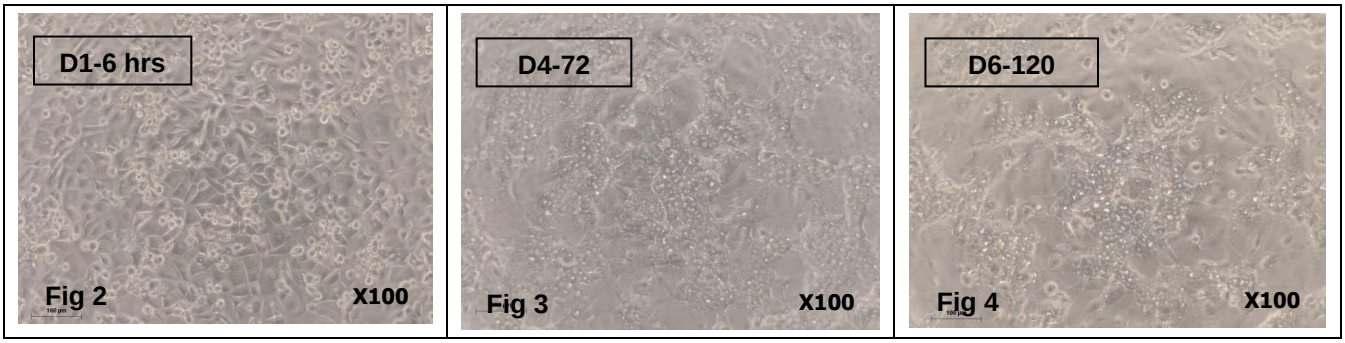
c. On days 5 and 7, replace the NoSpin HepaRG™ Pre-induction and Tox Medium.
d. On day 8, replace the NoSpin HepaRG™ Pre-induction and Tox Medium, and incubate the cells in monolayer with the test compounds according to the study protocol.

Table 6. Suggested timeline for toxicity studies at day 8

Day 1	Thaw and seed the cells using NoSpin HepaRG™ Thawing and Plating Medium.
Day 2 **24 hours**	Remove NoSpin HepaRG™ Thawing and Plating Medium, and replace with the Pre-induction and Tox Medium.
Day 5 **96 hours**	Replace the NoSpin HepaRG™ Pre-induction and Tox Medium.
Day 7 **144 hours**	Replace the NoSpin HepaRG™ Pre-induction and Tox Medium.
Day 8 **168 hours**	Replace the NoSpin HepaRG™ Pre-induction and Tox Medium and incubate the cells in monolayer with the test compounds according to your protocol.

VII. Cell Morphology

1. **Fig 2**: Six hours after plating, cells attach and spread to form a monolayer.
2. **Fig 3**: After 72-96 hours in culture, restructuring of the cell monolayer to a hepatocyte-like cell cluster organization can be observed.
3. **Fig 4**: 120 hours after plating, hepatocyte-like cells are organized in well-delineated trabeculae with many bright canaliculi-like structures.



VIII. Ordering Information

NoSpin HepaRG™ Cells

Cat. No.	Product	Size
NSHPRG	NoSpin HepaRG™ Cells, Cryopreserved	≥ 8 million viable cells

Media (purchased separately-see Table 2)

Cat. No.	Product	Size
MH100	NoSpin HepaRG™ Base Medium with Suplement	100 mL
MHTAP	NoSpin HepaRG™ Thawing and Plating Additive	11.8 mL
MHMET	NoSpin HepaRG™, Maintenance and Metabolism Additive	14 mL
MHPIT	NoSpin HepaRG™ Pre-Induction and Tox Additive	12.5 mL
MHIND	NoSpin HepaRG™Induction Additive (Serum Free)	0.6 mL

IX. Product Warranty

CULTURES HAVE A FINITE LIFESPAN *IN VITRO*. Lonza warrants its cells in the following manner only if recommended Media and Reagents are used.

X. Quality Control

For detailed information concerning QC testing, please refer to the Certificate of Analysis.

When placing an order or for Scientific Support, please refer to the product numbers and descriptions listed above. For a complete listing of all Cell Biology Products, refer to the Lonza website or our current catalog. To obtain a catalog, additional information or Scientific Support, you may contact Lonza by web, e-mail, telephone, fax or mail. Contact details are listed at the top of this document.

XI. Safety Statements

THESE PRODUCTS ARE FOR RESEARCH USE ONLY. Not approved for human or veterinary use, for application to humans or animals, or for use in clinical or *in vitro* procedures.

WARNING: PRIMARY CELL PRODUCTS CONTAIN HUMAN SOURCE MATERIAL, TREAT AS POTENTIALLY INFECTIOUS. All human sourced products should be handled at the Biological Safety Level 2 to minimize exposure of potentially infectious products, as recommended in the CDC-NIH Manual,

Biosafety in Microbiological and Biomedical Laboratories, 5th ed.
If you require further information, please contact your site Safety Officer or Scientific Support.