

## Product Information

### Primary Human Hepatocytes

#### LifeNet Health®

Catalog Number:

**MTOXH1000** - Cryoplateable Primary Human Hepatocytes

**MTOXH1001** - Cryopreserved Primary Human Hepatocytes Metabolism Qualified Suspension Grade

**MTOXH1002** - Cryoplateable Primary Human Hepatocytes Induction Certified

**MTOXH1005** - Cryopreserved Primary Human Hepatocytes Research Grade

Storage Temperature –150 °C or below  
in liquid nitrogen vapor phase

**MTOXH1008** - Cryopreserved Primary Human Hepatocytes High BMI

**MTOXH1011** - Cryopreserved Primary Human Hepatocytes Neonatal

**MTOXH1012** - Cryopreserved Primary Human Hepatocytes Juvenile

**MED-HHTM** - Human Hepatocyte Thawing Medium

**MED-HHPM** - Human Hepatocyte Plating Medium

**MED-HHCM** - Human Hepatocyte Culture Medium

**MED-HHPMSP** - Human Hepatocyte Plating Medium Supplement Pack

**MED-HHCMSP** - Human Hepatocyte Culture Medium Supplement Pack

## TECHNICAL BULLETIN

### Product Description

Primary human hepatocytes from LifeNet Health meet the specific needs of a wide range of scientific research applications. Donated liver tissues are procured under state-of-the-art conditions using the highest standards for tissue recovery and preservation, which utilize enhanced tissue handling and transportation methods, and minimize warm and cold ischemia time to optimize tissue processing outcomes. These measures, combined with refined cell isolation techniques and advanced post-thaw characterization, represent a new industry standard for hepatocyte quality and performance. Prior to release, each batch of cryopreserved hepatocytes is carefully characterized to determine the post-thaw results. The batch-specific functionality and donor data includes the following:

- Cell viability and yield per vial
- Morphological integrity and attachment efficiency
- Optimal seeding density based on 24 and 96 well plate formats
- CYP enzyme activity using prototype selective substrates

Each batch comes with a comprehensive Certificate of Analysis (CoA) with representative images, relevant donor demographics, BMI, pre-mortem liver function lab values, serological test results, and pertinent tobacco, alcohol, drug, and medication history.

Additional functional testing can be performed upon request for specific cell culture applications.

Liver biopsy histology images and pathological results may also be available upon request.

### Component

This product is a cryovial containing at least 5 million primary human hepatocytes.

### Reagents and Equipment Required but Not Provided.

#### Media and Supplements

Note: Neither media nor supplements are supplied with the vials. These must be obtained prior to receiving the vials.

- Human Hepatocyte Thawing Medium (HHTM) (Catalog Number MED-HHTM)
- Human Hepatocyte Plating Medium (HHPM) (Catalog Number MED-HHPM)
- Human Hepatocyte Culture Medium (HHCM) (Catalog Number MED-HHCM)
- Human Hepatocyte Plating Medium Supplement Pack (Catalog Number MED-HHPMSP)
- Human Hepatocyte Culture Medium Supplement Pack (Catalog Number MED-HHCMSP)

### Thawing and Plating

- 70% ethanol (prepared from Ethanol, Catalog Number E7148)
- Bio-Pure™ alcohol wipes (Catalog Number Z688487)
- Small laboratory ice tray
- Portable Dewar or other container to transport frozen vials in liquid nitrogen
- Tongs or forceps
- Ice
- Liquid nitrogen
- 37 °C water bath (operating range 35–38 °C)
- Centrifuge with 50 mL tube carriers
- 1,000 µL multichannel electronic pipette
- 1,000 µL multichannel electronic pipette tips
- Serological pipettor (i.e., Pipet-Aid®)
- 1, 2, 5, 10, and 25 mL serological pipettes
- Plastic or glass aspiration tips and vacuum aspiration system (optional)
- 50 or 100 mL reagent reservoir
- Collagen coated (rat tail, Type I) culture vessels (multiwell plates, dishes, etc.)
- Disposable plastic or washable glass media bottles (100–250 mL)
- Timer
- 50 mL conical tubes
- 1,000 µL micropipette
- 1,000 µL pipette tips
- 0.4% Trypan Blue solution (Catalog Number T8154)
- Hemocytometer and inverted microscope (or equivalent cell staining and counting device)

### In Situ Metabolism Assay

- Ultra-low attachment surface 24 well (with lid) flat bottom culture plate
- Appropriate solvent(s) and CYP probe substrates for *in situ* metabolism assay (see Appendix, Table 5).
- Appropriate internal standards for analytical analysis of substrates
- Orbital shaker (capable of staying in incubator for 60 minutes)
- Acetonitrile for UHPLC, for mass spectrometry (Catalog Number 900667)
- Deep well 1 or 2 mL sample block or plastic tubes
- Vortexor

### **Precautions and Disclaimer**

This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

### **Procedures**

#### **Thawing Procedure**

#### Preparation of Human Hepatocyte Thawing and Plating Media (HHTM & HHPM)

Note: At least one 50 mL bottle of Human Hepatocyte Thawing Medium (Catalog Number MED-HHTM) is needed per vial of cryopreserved human hepatocytes. Up to five (5) vials of cryopreserved human hepatocytes can be placed in one 50 mL conical tube with Human Hepatocyte Thawing Medium. DO NOT overload container of Human Hepatocyte Thawing Medium with excess cells.

1. Remove Human Hepatocyte Thawing Medium (HHTM) bottle(s) (Catalog Number MED-HHTM) from –20 °C storage and thaw protected from light either overnight at 4 °C or immediately prior to use. Note: One bottle can be used for up to 4–5 vials of cryopreserved human hepatocytes.
2. Remove Human Hepatocyte Thawing Medium Supplement Pack (Catalog Number MED-HHPMS) from –20 °C and thaw Components A-E either overnight at 4 °C or immediately prior to preparing the medium.
3. Remove Human Hepatocyte Plating Medium (HHPM) (Catalog Number MED-HHPM) from 4 °C and add Components A-E (see Appendix, Table 1 for amounts) to prepare Fully Supplemented HHPM.
4. Optional step: Filter Fully Supplemented HHPM through 0.2 µm filter.
5. Aliquot sufficient volume of Fully Supplemented HHPM for a single day's use (~20 mL per vial) into a sterile bottle.
6. Warm both HHTM and Fully Supplemented HHPM to 37 °C in a water bath (typically 20–30 minutes). Protect from light and do NOT warm for excessive periods of time.

#### Thawing Cryopreserved Human Hepatocytes

1. Refer to the batch specific CoA for average yield per vial as well as any special centrifugation instructions.
2. Wear personal protective equipment (PPE) appropriate for liquid nitrogen and human hepatocyte handling.
3. Fill a portable Dewar flask or other liquid nitrogen container with a small amount of liquid nitrogen (enough to submerge at least half of the vial). Note: A precharged liquid nitrogen dry shipper is typically the easiest and best method for transferring the vial(s).

4. Set up the following in a biological safety cabinet:
  - 50 mL conical tube
  - 1,000  $\mu$ L pipette with 1,000  $\mu$ L tips
  - Small laboratory ice tray containing ice
  - Aspiration tips
  - Serological pipettor with various serological pipette tips
5. Remove 50 mL bottle of HHTM from water bath, spray with 70% ethanol, and place in biological safety cabinet. Invert tube to mix thoroughly. Pour ~30 mL of HHTM into a 50 mL conical tube.

**Note:** The following steps should be completed **as quickly as possible** to maintain hepatocyte viability and functionality.

6. QUICKLY remove vial(s) with cryopreserved human hepatocytes from cryogenic storage freezer and place in the portable Dewar flask or liquid nitrogen dry shipper.
7. Carefully transfer cryopreserved cells in liquid nitrogen container to culture area.
8. QUICKLY remove vial(s) from liquid nitrogen (using tongs or forceps) and slightly loosen cap(s) to release pressure. Re-tighten cap(s).  
**Note:** Do NOT remove cap(s) or expose the contents to air!
9. QUICKLY submerge vial(s) vertically into 37 °C water bath, until ~0.5 cm below the cap (see Figure 1). Transferring vials from the liquid nitrogen, loosening caps, and submerging into water should take less than 10 seconds.

**Figure 1.**  
Correct placement of vial in water bath for thawing



**Note:** The vial is submerged to the top of the frozen cell suspension; however, the cap is above the water.

10. Thaw for 60–120 seconds – until a thin spindle of frozen cells remains in the center of the vial(s) (see Figure 2).

**Figure 2.**  
Thawed Cells



Remove vial from water bath when all ice is thawed except a thin spindle of frozen cells in the center of the vial.

11. QUICKLY clean cap and neck of vial with alcohol wipe, place in ice tray, and move tray to biological safety cabinet.  
**Note:** If thawing multiple vials, remove individual vials from the water bath and place on ice when properly thawed, regardless of the readiness of the remaining vial(s).
12. Moving at a quick pace and working with one vial at a time, remove vial cap and pour hepatocytes into the prepared 50 mL conical tube of HHTM.
13. Using 1,000  $\mu$ L pipette, rinse vial with 1,000  $\mu$ L of the HHTM-cell suspension and then transfer the rinse back into the 50 mL tube of HHTM. Repeat this step for all other vials (working quickly).
14. Once contents from all vials have been placed in the 50 mL conical tube of HHTM, fill to 50 mL with remaining fresh HHTM. Tighten cap on tube and gently invert tube three times to uniformly suspend hepatocytes.
15. Centrifuge hepatocytes at 100  $\times$  g for 8 minutes at room temperature.  
**Note:** Cells from individuals with a high BMI may have slightly different centrifugation conditions. Examine the CoA for the specific batch for any special instructions prior to centrifugation in order to maximize the yield.
16. Return the tube to the biosafety cabinet and gently vacuum aspirate the supernatant without disturbing the pellet at the bottom of the tube. Alternatively, gently and very carefully pour the supernatant into a waste container without disturbing or shaking the pellet.

17. Add 3–5 mL of Fully Supplemented HHPM (volume depends on size of pellet/average yield) and gently rock the conical tube to resuspend hepatocytes. Do NOT resuspend cells using a pipette or by vortexing. Note: Typical concentration for cell counting should be  $1\text{--}2 \times 10^6$  cells/mL to allow for accurate Trypan Blue or automated counter analysis. Average yield per vial is stated on the CoA for each batch.
18. Count hepatocyte suspension to determine yield and viability by using the Trypan Blue exclusion test and/or AO/PI staining on a hemocytometer or automated cell counter. Note: Automated cell counters are not ideal for hepatocyte counting unless a hepatocyte-specific program is used. Accurate concentration of the cell suspension is critical for proper plating. Use a manual counting method if a hepatocyte-specific program is not available.
19. Proceed to plating cells or using them in suspension.

### Cell Plating Procedure

#### Preparation of Human Hepatocyte Plating and Culture

##### Media

Ensure non-expired Fully Supplemented Human Hepatocyte Plating Medium (HHPM) is available (~15 mL per plate). This media preparation may be performed immediately prior to thawing hepatocytes.

1. Remove Human Hepatocyte Culture Medium (HHCM) Supplement Pack (Catalog Number MED-HHCMSP) from  $-20\text{ }^{\circ}\text{C}$  freezer and thaw Components A–C. This can be done overnight at  $4\text{ }^{\circ}\text{C}$  or at  $37\text{ }^{\circ}\text{C}$  immediately prior to preparing the medium.
2. Remove Human Hepatocyte Culture Medium (HHCM) (Catalog Number MED-HHCM) from  $4\text{ }^{\circ}\text{C}$  refrigerator and add Components A–C (see Appendix, Table 2 for amounts). Mix well after adding components by gently inverting container several times. Optional: Filter Fully Supplemented HHCM through  $0.2\text{ }\mu\text{m}$  filter. Note: Fully Supplemented HHCM should be prepared fresh daily for best results. If not prepared daily, use within 72 hours.
3. Aliquot sufficient volume of Fully Supplemented HHCM for a single day's use (~15 mL per standard multiwell plate) into a sterile bottle or conical tube.
4. Warm both Fully Supplemented HHPM and Fully Supplemented HHCM to  $37\text{ }^{\circ}\text{C}$  in a water bath (typically 20–30 minutes). Protect from light and do NOT warm for excessive periods of time.

#### Plating of Thawed Cryopreserved Human Hepatocytes

1. Place Fully Supplemented HHPM in  $37\text{ }^{\circ}\text{C}$  water bath prior to thawing cryopreserved human hepatocyte vials until temperature is  $37\text{ }^{\circ}\text{C}$  (typically 20–30 minutes).
2. Follow the Procedure for Thawing Cryopreserved Human Hepatocytes. After thawing, refer to the CoA for each batch of cryopreserved human hepatocytes for recommended seeding density for standard collagen-coated 24 well plates. Note: The seeding density is unique to each batch for cryopreserved human hepatocytes. A range of densities is provided in Appendix, Table 3, but the actual final density for a specific batch is provided in the CoA. If not using 24 well plates, determine the appropriate cell density per batch by testing different densities (refer to Appendix, Table 3 for ranges).
3. Determine the final cell density and volume required for application. If the final volume required is greater than 50 mL, transfer the cell stock from the 50 mL conical tube to an appropriately sized sterile bottle. Add Fully Supplemented HHPM to cell stock until desired final cell density is reached.
4. Ensure the suspension is homogeneously mixed by gentle rocking or inverting.
5. Pour cell suspension into the sterile reagent reservoir until half full.
6. Using an electronic multichannel pipette, transfer cell suspension from reagent reservoir to culture vessels. Note: Hepatocytes settle out of suspension quickly. Gently rock the reservoir in all directions to ensure the cell suspension is homogenous when refilling pipette. Likewise, mix the cell suspension in the conical tube or bottle prior when refilling the reservoir.
7. Remove culture vessels from the biological safety cabinet in stacks of 1-5 and place in an incubator ( $37\text{ }^{\circ}\text{C}$ , 5%  $\text{CO}_2$ ).
8. Shake/rock culture vessels in an up and down/left to right manner on a solid surface, three times on the incubator shelf. Repeat this motion at 15 minute intervals for the first 60 minutes of culture. This shaking should be gentle yet vigorous enough to displace cells grouped in the center of each well.
9. Warm Fully Supplemented Human Hepatocyte Culture Medium (HHCM) to  $37\text{ }^{\circ}\text{C}$  in an appropriate water bath at 3.5–5.5 hours post plating. Note: Aliquot and warm only the amount of medium needed to add to the number of plates seeded (see Appendix, Table 4). Do not warm or leave the medium in water bath for longer than necessary.

10. At 4–6 hours post plating, gently shake plates and remove Fully Supplemented Human Hepatocyte Plating Medium (HHPM) by gentle vacuum aspiration and replace with Fully Supplemented HHCM.
  - a. Remove plates from the incubator and place in the biological safety cabinet (no more than 5 culture plates at one time).
  - b. Gently shake plates in an up and down/left to right manner on a solid surface, to dislodge dead cells.
  - c. Tilt plates and gently vacuum aspirate the medium from the side of each well without touching the cell monolayer.
  - d. Using a serological, micro-, or electronic multichannel pipette add Fully Supplemented HHCM in volumes shown in Appendix, Table 4.
11. Return plates to incubator for overnight or until experimental protocol dictates.
12. If warranted, overlay cells with extracellular matrix the next morning.
13. Replace medium with fresh warm Fully Supplemented HHCM daily.

### ***In Situ* Metabolism Determination for Cryopreserved Human Hepatocytes in Suspension**

#### Preparation of Suspension Culture Substrates

1. Prepare all substrate stock solutions to be 2× Working Substrate Solution in Fully Supplemented HHCM (See Appendix, Table 5 for standard CYP probe substrates.)
2. Calculate the minimum volume of 2× Working Substrate Solution required for each substrate by multiplying the number of wells by the volume of substrate solution required per well. Include 10% overage volume to cover for possible pipette error.
3. Add time-matched substrate solutions into adjacent wells of appropriate-sized multiwell plate(s) according to the assay plate map (this will be termed the “substrate plate” in subsequent steps).
4. Set up CO<sub>2</sub> incubator at 36.5–37.5 °C. Place the orbital shaker and substrate plate in the incubator and set the shaker to 150 rpm.

#### Suspension Culture Substrate Incubations

1. Calculate the number of cells required for each study using a 24 well plate, using the following equation as a guide:

[Number of substrates (not including 7-HC or 7-EC) × (cells/mL × 0.25 mL/well) × number replicates] +

[Number of 7-HC and/or 7-EC substrates × (cells/mL × 0.5 mL/well) × number replicates]

= Number of cells required

Example: For a test of phenacetin, testosterone, and 7-HC (3 substrates) with 3 replicates and a cell concentration of  $2 \times 10^6$  cells/mL

$[2 \times (2 \times 10^6 \text{ cells/mL} \times 0.25 \text{ mL}) \times 3] + [1 \times (2 \times 10^6 \text{ cells/mL} \times 0.5 \text{ mL}) \times 3] = 6 \times 10^6 \text{ cells required}$

Note: A total of  $\sim 12 \times 10^6$  cells are required for each multiwell plate; therefore the number of vials of cryopreserved hepatocytes required will vary from batch to batch. Refer to the CoA for the average yield per vial for a particular batch prior to starting experiments.

2. Thaw hepatocytes as indicated in Thawing Cryopreserved Human Hepatocytes Protocol section, with the modification to resuspend the cells post-centrifugation in Fully Supplemented Human Hepatocyte Culture Medium (HHCM), NOT Human Hepatocyte Plating Medium (HHPM).
3. Dilute cells to  $2 \times 10^6$  cells/mL using Fully Supplemented HHCM.
4. Add 250  $\mu$ L cell suspension (for use with most substrates) or 500  $\mu$ L cell suspension (for use with 7-hydroxycoumarin and 7-ethoxycoumarin substrates) into respective wells of a 24 well ultra-low attachment plate and place plate onto orbital shaker in incubator for 10 minutes.
5. Remove cell and substrate plates from the incubator. Transfer the 2 $\times$  Working Substrate solutions from the substrate plate into the appropriate wells of the cell plate to start reactions.  
Note: Time-matched substrate solutions can be added using a multichannel pipettor.
6. Replace cell plate with added substrates onto the orbital shaker (150 rpm) inside the incubator at 37 °C and 5% CO<sub>2</sub> and allow plate to incubate for the appropriate times required per substrate (see Appendix, Table 5).
7. At the end of the incubation period, terminate reactions by adding an equal volume of ice-cold acetonitrile (ACN) containing the internal standard.
8. Transfer reaction-terminated samples from cell plate to tubes or deep-well sample block and vortex for 30 seconds at high speed.
9. Centrifuge samples at 1000  $\times$  g for 10 minutes at 4 °C.
10. Transfer supernatants (typically  $\geq 150$   $\mu$ L) to new tubes or deep-well sample block and process immediately or freeze at –80 °C until analysis by LC/MS/MS.

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Bio-Pure is a trademark of Diversified Biotech.  
Pipet-Aid is a registered trademark of Drummond Scientific Co.

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## Appendices

**Table 1.**  
Supplementing Human Hepatocyte Plating Medium (HHPM)

Base HHPM Medium SKU Size	250 mL	100 mL	50 mL
Component A	12.5 mL	5 mL	2.5 mL
Component B	2.5 mL	1 mL	0.5 mL
Component C	2.5 mL	1 mL	0.5 mL
Component D	25 $\mu$ L	10 $\mu$ L	5 $\mu$ L
Component E	250 $\mu$ L	100 $\mu$ L	50 $\mu$ L

**Table 2.**  
Supplementing Human Hepatocyte Culture Medium (HHCM)

Base HHCM Medium SKU Size	500 mL	250 mL	150 mL	100 mL	50 mL	25 mL
Component A	5 mL	2.5 mL	1.5 mL	1 mL	0.5 mL	0.25 mL
Component B	5 mL	2.5 mL	1.5 mL	1 mL	0.5 mL	0.25 mL
Component C	5 $\mu$ L	2.5 $\mu$ L	1.5 $\mu$ L	1 $\mu$ L	0.5 $\mu$ L	0.25 $\mu$ L

**Table 3.**  
Seeding Densities for Cryopreserved Human Hepatocytes

Culture Vessel	Seeding Density (cells × 10 <sup>6</sup> /mL)	Volume per Well
6 well plate	0.9–1.1	2 mL
12 well plate	0.8–1.0	1 mL
24 well plate	0.7–1.0	500 µL
48 well plate	0.6–0.8	250 µL
96 well plate	0.6–0.8	100 µL

**Table 4.**  
Volumes of Fully Supplemented Human Hepatocyte Culture Medium for Plating

Culture Vessel	Volume per Well
6 well plate	1.5 mL
12 well plate	800 µL
24 well plate	300 µL
48 well plate	200 µL
96 well plate	75 µL

**Table 5.**  
Prototypical Suspension Culture Substrate Probes and Incubation Times

CYP Activity	Substrate	Working Solution Concentration (2×, µM)	Final Concentration (1×, µM)	Incubation Times (minutes)	Market Metabolite
CYP1A2	Phenacetin	200	100	15	4-Acetamidophenol (APAP)
CYP2A6	Coumarin	10	5	15	7-Hydroxycoumarin (7-HC)
CYP2B6	Bupropion	1000	500	15	Hydroxybupropion
CYP3A4 #1	Testosterone	400	200	15	6β-Hydroxytestosterone
CYP3A4 #2	Midazolam	20	10	10	1-Hydroxymidazolam
CYP2C8	Paclitaxel	40	20	30	6α-Hydroxypaclitaxel
CYP2C9	Tolbutamide	50	25	15	4-Hydroxytolbutamide
CYP2C19	(S)-Mephenytoin	500	250	30	4-Hydroxymephenytoin
CYP2D6	Dextromethorphan	30	15	15	Dextrorphan
CYP2E1	Chlorzoxazone	500	250	15	6-Hydroxychlorzoxazone
Phase I (ECOD)	7-Ethoxycoumarin (7-EC)	200	100	30	7-Hydroxycoumarin (7-HC)
Phase II (7-HCG)	7-Hydroxycoumarin (7-HC)	200	100	30	7-Hydroxycoumarin glucuronide
Phase II (7-HCS)	7-Hydroxycoumarin (7-HC)	200	100	30	7-Hydroxycoumarin sulfate