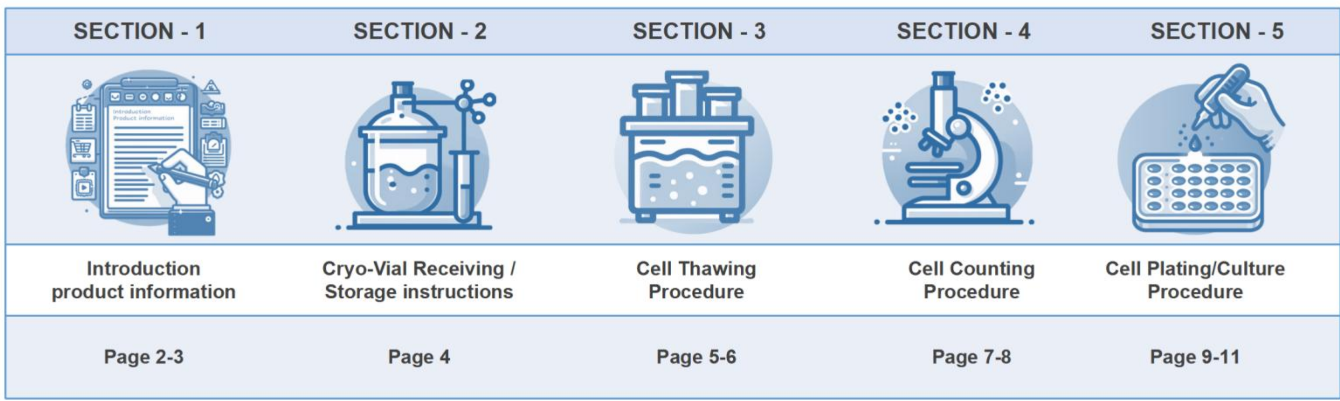
User Guide:
Thawing and Plating Cryopreserved Hepatocytes

Procedural Sections Overview



SECTION - 1	SECTION - 2	SECTION - 3	SECTION - 4	SECTION - 5
Introduction product information	Cryo-Vial Receiving / Storage instructions	Cell Thawing Procedure	Cell Counting Procedure	Cell Plating/Culture Procedure
Page 2-3	Page 4	Page 5-6	Page 7-8	Page 9-11

PRECAUTIONS: Cells should be handled following biosafety level 2 procedures. Universal precautions should be utilized when working with these cells. Use aseptic techniques when thawing or handling the cells.

SECTION – 1: INTRODUCTION & PRODUT INFORMATION

INTRODUCTION

This user manual offers comprehensive guidance for the thawing and preparation of cryopreserved hepatocytes, tailored for use in suspension and plateable hepatocyte assays. The instructions provided are in accordance with the pre-qualified cryopreserved hepatocyte grade indicated in the Certificate of Analysis (COA) for the specific product batch.

Designed for the thawing process of both human and animal cryopreserved hepatocytes, this user guide underscores the importance of thoroughly reviewing the entire protocol before initiating the procedure. Hepatocyte viability and functionality are highly dependent on strict adherence to the protocol. Whether working with human or animal cryopreserved hepatocytes, it is essential to follow the provided guidelines for thawing, counting, and plating to ensure optimal hepatocyte functionality throughout the entire experimental duration.

CRYOPRESERVED HEPATOCYTE PRODUCT INFORMATION

Cryopreserved hepatocytes from various species, prepared by MileCell, meet the specific needs of diverse scientific research applications. Each batch of cryopreserved hepatocytes undergoes thorough characterization to assess post-thaw cell quality.

- ✓ Post-thaw cell viability and yield
- ✓ Morphological integrity evaluation
- ✓ Thawed cell attachment efficiency examination
- ✓ Phase I & II enzyme activity using specific substrates.

Each batch is accompanied by a comprehensive Certificate of Analysis (CoA), featuring representative images, donor details, and safety data.

Table 1: Animal cryopreserved hepatocytes products

Cat. No.	Product Description	Size
Suspension Hepatocytes		
CMH-100CB-SQ	Cryopreserved Male C57BL/6 Mouse Hepatocytes (Pooled, Suspension & Metabolism Qualified)	5 million/vial
CMH-100CD-SQ	Cryopreserved Male CD-1 Mouse Hepatocytes (Pooled, Suspension & Metabolism Qualified)	5 million/vial
CRH-100SD-SQ	Cryopreserved Male SD Rat Hepatocytes (Pooled, Suspension & Metabolism Qualified)	5 million/vial
CRH-100WH-SQ	Cryopreserved Male Wistar Han Rat Hepatocytes (Pooled, Suspension & Metabolism Qualified)	5 million/vial
CMH-100GPP-SQ	Cryopreserved Male Hartley Guinea Pigs Hepatocytes (Pooled, Suspension & Metabolism Qualified)	5 million/vial
CDH-100BE-SQ	Cryopreserved Male Beagle dog Hepatocytes (Pooled, Suspension & Metabolism Qualified)	5 million/vial
CCH-100CYP-SQ	Cryopreserved Male Cynomolgus Monkey Hepatocytes (Pooled, Suspension & Metabolism Qualified)	5 million/vial
CRH-100NRP-SQ	Cryopreserved Male New Zealand Rabbit Hepatocytes (Pooled, Suspension & Metabolism Qualified)	5 million/vial
CCH-100FCP-SQ	Cryopreserved Male Felis Catus Hepatocytes (Pooled, Suspension & Metabolism Qualified)	5 million/vial
Plateable Hepatocytes		
CMH-100CBP-PQ	Cryopreserved Male C57BL/6 Mouse Hepatocytes (Pooled, Plateable Qualified)	5 million/vial
CMH-100CDP-PQ	Cryopreserved Male CD-1 Mouse Hepatocytes (Pooled, Plateable Qualified)	5 million/vial

CRH-100SDP-PQ	Cryopreserved Male SD Rat Hepatocytes (Pooled, Plateable Qualified)	5 million/vial
CRH-100WHP-PQ	Cryopreserved Male Wistar Han Rat Hepatocytes (Pooled, Plateable Qualified)	5 million/vial
CDH-100BES-PQ	Cryopreserved Male Beagle dog Hepatocytes (Single, Plateable Qualified)	5 million/vial
CDH-100BEP-PQ	Cryopreserved Male Beagle dog Hepatocytes (Pooled, Plateable Qualified)	5 million/vial
CCH-100CYS-PQ	Cryopreserved Male Cynomolgus Monkey Hepatocytes (Single, Plateable Qualified)	5 million/vial
CRH-100NRS-PQ	Cryopreserved Male New Zealand Rabbit Hepatocytes (Single, Plateable Qualified)	5 million/vial
CCH-100FCS-PQ	Cryopreserved Male Felis Catus Hepatocytes (Single, Plateable Qualified)	5 million/vial

Custom Hepatocyte Options:

In addition to our standard offerings, MileCell also offers customizable hepatocyte solutions tailored specifically to your requirements, including choices of species, gender (male, female, mixed), and donor quantity. Contact us to learn more about how we can support your research endeavors.

PURPOSE This section of the user guide provides guidance and essential recommendations for the secure reception and storage of cryopreserved hepatocytes. The primary purpose is to emphasize the importance of transferring the cells from the cryoshipper to a temperature-controlled cryogenic freezer upon delivery. This ensures the preservation of their characteristics and functionality while minimizing the risk of product degradation.

MATERIALS / EQUIPMENTS REQUIRED

Materials Required for Section 2 Procedure:
• Appropriate personal protective equipment (PPE)
• Insulated container (such as a foam box) for holding liquid nitrogen and a cryovial box
• Tongs or forceps
• Cryogenic storage tank (capable of maintaining temperatures ≤ -150°C)
• Cryovial storage box

CRYO-VIAL RECEIVING & STORAGE INSTRUCTIONS

1. **Personal Protective Equipment (PPE):**
   - Wear appropriate PPE for handling liquid nitrogen and cryovials, including a face shield, antifreeze gloves, shoe covers and lab coat.
2. **Preparation of foam box & Cryovial transfer:**
   - Fill the foam box with approximately half its capacity with liquid nitrogen.
   - Remove cryovials from the cryoshipper and promptly place them into the foam box with liquid nitrogen.
3. **Cryovial box placement:**
   - Immediately use tongs to transfer the vials to a cryovial box submerged in liquid nitrogen within the foam box.
4. **Cryogenic storage:**
   - Quickly place the filled cryovial box into the designated rack within your cryogenic storage tank at temperatures either < -150°C in the vapor phase of liquid nitrogen or -196°C in liquid nitrogen.
   - In the case of larger quantities of vials shipped in boxes, immediately position these boxes directly into the cryogenic storage tank.
5. **Return shipment for the cryoshipper:**
   - After placing all vials in the cryogenic storage tank, follow the provided instructions for the shipment process to return the liquid nitrogen vapor shipping dewar.

SECTION – 3: CELL THAWING PROCEDURE

PURPOSE

This section provides instructions and recommendations for the appropriate thawing cryopreserved primary hepatocytes, ensuring optimal integrity and quality of the thawed cells for standard ADME/Tox applications.

REAGENTS AND MATERIALS REQUIRED

Reagents & Materials Required for Section 3 Procedure	
• Biological safety cabinet (BSC)	• Portable liquid nitrogen dewar for transporting vials
• Personal protective equipment	• 37°C water bath
• 75% alcohol and lab wipes	• Centrifuge with 50 mL tube carriers
• Laboratory ice tray with ice	
• Tongs, forceps, Timer	• Hepatocyte Thawing Medium Kit (HTM-500K)*
• 100μL, 200μL, 1000 μL pipettes & tips	• Hepatocyte Plating Medium Kit (HPM-500K)*
• Pipet-Aid & 1, 2, 5, 10, 25 mL sterile pipettes	• Hepatocyte Maintenance Medium Kit (HMM-500K)*
• 50 mL conical centrifuge tubes	

** Note 1: Prepare one 50 mL conical tube with approximately 40 mL of complete HTM medium per lot of cryopreserved hepatocytes. Thawing up to 3 cryovials in a single HTM tube is recommended to prevent overloading with excess cryovial-cells.*

**Note 2: Hepatocyte Thawing Medium Kit (HTM) ,Hepatocyte Plating Medium Kit (HPM) & Hepatocyte Maintenance Medium Kit (HMM) required for this section are available for additional purchase from MileCell. You can also source these media from homemade preparations or reputable suppliers, following their specific usage instructions.*

**Note 3: It is crucial to verify and prepare all necessary media before initiating the hepatocyte thawing and plating procedures. Please follow the specific instructions provided by the supplier(s) if using commercially available media.*

CELL THAWING PROCEDURE

1. Fill a portable dewar with an ample amount of liquid nitrogen. Retrieve the required cryovial(s) from the liquid nitrogen storage tank and promptly submerge the vial(s) into the portable dewar. Place the portable dewar beside the water bath.
2. Warm 50 mL centrifuge tube(s) containing complete Hepatocyte thawing medium in a 37°C water bath for approximately 30 minutes.

Note: Generally, a single 50 mL centrifuge tube containing approximately 40 mL of thawing medium is sufficient for thawing a maximum of 3 vials. However, it is recommended to follow the thawing medium provider's instructions for optimal results.

3. After warming the thawing medium, disinfect the tube(s) using a 75% ethanol wipe and place it in the biological safety cabinet (BSC).

IMPORTANT! Perform all the following steps quickly to ensure the preservation of hepatocyte viability and integrity.

4. Quickly remove a cryovial from the liquid nitrogen drawer using metal forceps. Slightly loosen the cap by 1/4 turn to release pressure ,then retighten.
5. Submerge the cryovial vertically into a 37°C water bath, ensuring the cap remains above the waterline.

6. Gently shake the cryovial in water bath for about 2 minutes. Remove the vial from the water bath when only a small visible ice core remains. Disinfect the cryovial surface with a 75% alcohol wipe and promptly transfer it to a BSC.

   Note: If thawing multiple vials, thaw and remove individual vials from the water bath and place them briefly on an ice tray.

7. Rapidly transfer the cryovial contents into a 50 mL conical tube containing pre-warmed thawing medium.
8. Pipette 1 mL of thawing medium to rinse the inner wall of the cryovial (2 times). Transfer the rinsed medium to the centrifuge tube as mentioned in step 7.
9. Tighten the centrifuge tube cap, gently invert the tube twice to mix the cell suspension.
10. Centrifuge at room temperature following the suggested conditions for the specific cell species. If using a commercial thawing medium, please adhere to the instructions provided by the supplier, ensuring the centrifuge's brake mode is set to off.

Table 2 outlines the suggested standard centrifugation speed and time

Species	Centrifugation speed (g)	Duration (min)
Mouse, Rat	50	2
Dog, Monkey	180	1

11. After centrifugation, return to the BSC and carefully discard the supernatant using a sterile pipette (avoid pouring directly to prevent cell loss). Retain approximately 0.5 mL of supernatant, ensuring not to disturb the cell pellet at the bottom of the tube.
12. Add 3mL of the appropriate medium based on the hepatocyte lot grade (e.g., Hepatocyte Maintenance Medium Kit (HMM) for suspension hepatocyte lot, Hepatocyte Plating Medium Kit (HPM) for plateable hepatocyte lot.

    Note: Refer to the COA for the thawed yield and aim for a typical concentration of about 2 x 10^6 cells/mL for accurate trypan blue staining assay.

13. Resuspend the cells by gently tapping or rocking the centrifuge tube. Adjust the cell suspension volume to 3 mL and briefly keep the suspended cells at 4°C (not exceeding 1 hour).

    Note: Do not shake during the cell resuspension steps.

14. Conduct a prompt cell counting procedure using Trypan Blue staining (refer to the procedure outlined in the following section 4) or AO/PI staining to assess both cell viability and the quantity of viable cells.

SECTION – 4: CELL COUNTING PROCEDURE

PURPOSE

This section provides detailed instructions and recommendations for assessing post-thaw cell viability and yield using the Trypan Blue Exclusion Method.

Principle

The Trypan Blue Exclusion Method quantifies viable cells in a suspension by specifically staining dead cells. It relies on the impermeability of living cells to Trypan Blue dye, leaving them unstained, whereas dead cells with permeable membranes absorb the dye, resulting in a blue cytoplasm. Microscopic analysis reveals the percentage of live cells in the total count, providing insights into overall cell viability.

REAGENTS AND MATERIALS REQUIRED

	Reagents & Materials Required for Section 4 Procedure	
	• Personal protective equipment	• Inverted phase contrast microscope
	• Laboratory ice tray with ice	• 75% alcohol and lab wipes
	• 20µL, 100µL, 200µL pipettes & tips	• 0.4% Trypan Blue solution (eg. Gibico, 15250-061)
	• Pipet-Aid & 1, 2, 5, 10 sterile pipettes	• Phosphate-buffered saline (PBS, 1X, pH 7.2)
	• Microcentrifuge tube (1.5-2mL)	• Hepatocyte Maintenance Medium Kit (HMM-500K)*
	• Hemocytometer & Cover Slips	• Hepatocyte Plating Medium Kit (HPM-500K)*
	• Manual steel cell counter	
	*Note 1: Hepatocyte Maintenance Medium Kit (HMM) without serum and red phenol is typically employed for hepatocyte suspension assays and is available for additional purchase from MileCell. Users have the flexibility to use a homemade medium tailored to their study conditions or choose a commercially available option . *Note 2: Hepatocyte Plating Medium Kit (HPM) for plateable hepatocyte lots is available for additional purchase from MileCell. You can also use relevant media from either homemade sources or different trusted suppliers.	

IMPORTANT! Perform the following section 4 procedure quickly to ensure the preservation of hepatocyte viability and functionality.

COUNTING PROCEDURE

Cell Counting with a Hemocytometer

1. Set up a hemocytometer with the coverslip forming two counting chambers (upper and lower) side view.
2. Prepare a 0.2% trypan blue solution by mixing 200 µL of 0.4% trypan blue solution with 200 µL of PBS in a microcentrifuge tube.
3. In a clean microcentrifuge tube, mix 100 µL of 0.2% Trypan Blue Solution with 100 µL of a well-mixed hepatocyte suspension obtained from section 3, resulting in a 2-fold dilution.

Note: For different dilutions, pre-diluted cells in HMM or HPM are acceptable, ensuring the final Trypan Blue concentration is 0.1%.

4. Take 20 µL of the Cell-Trypan blue mixture and gently load it onto each side of the hemocytometer. Ensure the area under the coverslip (approximately 5-10 µL) is filled via capillary action.
5. Before counting, quickly inspect the quadrants under the microscope to ensure even cell distribution. If uneven or containing bubbles, reload the hemocytometer.
6. Immediately, under a magnification inverted microscope (10x), count viable (bright cells) and dead cells (stained blue) in the four corner quadrants of the hemocytometer, assisted by a manual cell counter.

Note: To prevent extended exposure to trypan blue, restrict the counting period to within 5 minutes.
Note: Count cells in all four quadrants on one side of the hemocytometer, including two edges and excluding two edges along quadrant lines.

7. If necessary, repeat counting steps 4 to 7 on the second side of the hemocytometer to achieve cell counting on a total of 8 quadrants, enhancing counting accuracy.
8. Record the counting results:

Cell counting items	Recorded counting data.
Cell dilution factor	

Number of quadrants counted	
Total viable cells (bright cells)	
Total dead cells (blue cells)	
Total cell count (Living + Dead cells)	
Thawed cell vial quantities	

9. Calculate the cell viability, concentration and yield using the following formula:

Cell viability (%) = $\frac{\text{\# total live cells counted}}{\text{\# total cells counted (live + dead cells)}}$ x 100 = **% cell viability**

Cell concentration (viable cells/mL) = $\frac{\text{\# total live cells counted}}{\text{\# quadrants counted}}$ x dilution factor x 10 000 = **Viable cell number/ mL**

For example, following a 1:1 dilution of the cell sample with Trypan Blue (resulting in a dilution factor of 2), if you count 500 cells across all four corner quadrants, then:

Cell concentration (viable cells/mL) = $\frac{500}{4}$ x 2 x 10 000 = **2.5×10^6 cells/ mL**

Cell yield (viable cells/ vial) = cell concentration (viable cells/mL) x current cell volume (mL) = **total viable cells/vial**

For example, to calculate the total number of viable cells in your original cell suspension prepared from the thawed vial, multiply the cell concentration by the total volume of the cell suspension. If your initial sample volume is 3 mL:

Total viable cell yield in thawed vial = $2.5\times10^6\times3$ mL = **7.5×10^6 viable cells**

SECTION – 5: CELL PLATING / CULTURE PROCEDURE

PURPOSE

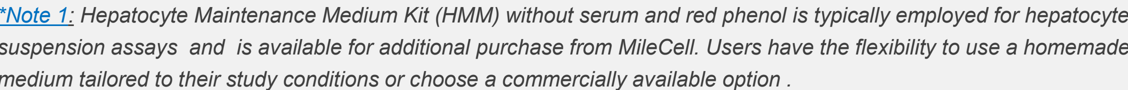
After thawing and counting the cells, this section provides instructions and recommendations for the optimal seeding of plateable-grade cells to achieve optimal confluency in monolayer cultures for long-term plated assays, and suspension-grade cells designed for short-term suspension assays.

Below are examples of applications suitable for both suspension and plateable hepatocytes:

Suspension Hepatocyte Lots are Appropriate for:	Plateable Hepatocyte Lots are Suitable for:
• Short incubation time (2-6 hours)	• 3-7 days in plated cell culture
• Metabolic screening assays	• CYP induction assays for drug-drug interaction studies
• In vitro intrinsic clearance/metabolic stability	• CYP inhibition assays
• Metabolite identification	• Metabolic stability and metabolite identification
• CYP inhibition	• Uptake Drug Transporter studies
• Uptake drug transporter assays	• Sandwich-Cultured Hepatocytes for efflux drug transporter and DDI studies
•	• Hepatocytes 3D spheroids for long-term metabolism study and disease modeling
•	*DDI: Drug-Induced Liver Injury*

REAGENTS AND MATERIALS REQUIRED

	Reagents & Materials Required for Section 5 Procedure	
	• Personal protective equipment	• Sterile glass media bottles (100-250 mL)
	• 70% alcohol and lab wipes	• Hepatocyte Plating Medium Kit (HPM-500K)*
	• Laboratory ice tray with ice	• Hepatocyte Maintenance Medium Kit (HMM-500K)*
	• Multichannel pipette (200µl, 1000µL)	• Collagen I-coated plate (6-, 12-, 24-, 48-, 96-, 384-Well)*
	• 100µL, 200µL 1000µL pipettes & tips	• Inverted phase contrast microscope
	• Pipet-Aid & 1, 2, 5, 10 sterile pipettes	• Cell culture incubator (37°C and 5% CO_2, 95% humidity)



**Note 1: Hepatocyte Maintenance Medium Kit (HMM) without serum and red phenol is typically employed for hepatocyte suspension assays and is available for additional purchase from MileCell. Users have the flexibility to use a homemade medium tailored to their study conditions or choose a commercially available option .*

**Note 2: Hepatocyte Plating Medium Kit (HPM) needed for this section is available for additional purchase from MileCell. You can also use the relevant mediums from homemade sources or different trusted suppliers.*

**Note 3: Collagen I-coated plates required for this procedure are available for additional purchase from MileCell. It is recommended to prepare them in advance within the laboratory or procure the necessary plate from homemade sources or reputable suppliers.*

IMPORTANT: It is advisable to promptly proceed with the steps outlined in Section 5 after cell thawing and counting to minimize the risk of cell degradation during the short storage period of the thawed cell suspension.

CELL PLATING / CULTURE PROCEDURE:

Procedure for Plateable Hepatocyte Lot

(refer to the COA of the thawed cell lot)

1. Before proceeding with cell plating, verify or prepare the reconstituted Hepatocyte Plating and Maintenance media (HPM, HMM) by combining supplements and the base medium according to the established home protocol or supplier instructions.
2. Calculate the required volume of complete media for cell plating and maintenance, aiming for approximately 15 mL per multi-well plate.
3. Pre-warm both HPM and HMM to 37°C in a water bath for approximately 20-30 minutes, avoiding prolonged warming periods.
4. Verify and unpack the necessary quantity and type of collagen-coated multi-well plates according to your study plan design.
5. Determine the total cell seeding volume, incorporating a 20% surplus volume, and choose the required cell density based on your specific experimental plate design, species, and plate format, as suggested in the table below:

Table 3: Seeding density range for cryopreserved hepatocytes by species and plate format

Culture Plate	Cell Seeding Density (x 10^6 cells / mL)				Cell Seeding Volume (mL/well)
	Mouse	Rat	Dog	Monkey	
96-well plate	0.19～0.24	0.25～0.32	0.29～0.36	0.32～0.40	0.1 mL
48-well plate	0.25～0.30	0.33～0.40	0.38～0.48	0.44～0.55	0.25 mL
24-well plate	0.24～0.30	0.32～0.40	0.40～0.50	0.51～0.63	0.5 mL
12-well plate	0.20～0.30	0.20～0.30	0.40～0.50	0.40～0.50	1.0 mL
6-well plate	0.32～0.40	0.38～0.48	0.49～0.61	0.70～0.80	2.0 mL

Note: The seeding density ranges provided are for standard cryoplateable animal hepatocytes. Slight adjustments in seeding density for each lot may be necessary to achieve optimal monolayer confluence.

6. Measure and transfer the thawed cell stock solution from the 50 mL centrifuge tube into a sterile glass bottle. Add the appropriate volume of hepatocyte plating medium to achieve the desired cell seeding density according to the determined cell concentration from section 4.
7. Gently mix the diluted cell suspension and use a multi-channel pipette to distribute the appropriate volume from a suitable reservoir to the collagen I-coated cell culture plates.
8. Quickly transfer the seeded plates (6-well, 12-well, 24-well, or 48-well) into a CO_2/37°C incubator. Shake the culture plates immediately using a "cross" or "North-South, East-West pattern" (↕, ↔) on the incubator shelf to ensure an even distribution of cells across the wells.

Note: For 96-well plates, refrain from shaking after seeding with hepatocytes. Gently transfer the seeded plate directly into a CO_2/37°C incubator. Shaking is not necessary for the 96-well format upon incubation.

9. Allow the cells to adhere for 5-6 hours (mouse and rat hepatocytes) or 12-16 hours (dog, monkey, and human hepatocytes). Observe cell morphology and attachment efficiency under a microscope and capture images.

10. Rat and mouse hepatocytes are typically ready for experimental use 5-6 hours post-attachment, whereas dog, monkey, and human hepatocytes require 12-16 hours. Additionally, for diverse applications, the plated hepatocyte cultures can be adapted into a sandwich-cultured model following the Matrigel supplier's guidelines.
11. Once cells have firmly attached, replace the Hepatocyte Plating Medium (HPM) with Hepatocyte Maintenance Medium (HMM) or overlay with Matrigel, tailored to your experimental requirements. Proceed with additional experiments as needed.
12. Change the cell culture medium completely every two days until the cells are used for the designated experiments. Refer to the table above and use the recommended seeding volumes for Hepatocyte Maintenance Medium (HMM) corresponding to the multi-well plate format.

Procedure for Suspension Hepatocyte lot

(refer to the COA of the thawed cell lot)

1. Ensure Hepatocyte Maintenance Medium (HMM), tailored to your specific assay, is prepared and verified. This medium can be sourced in-house or obtained from commercial suppliers.
2. Transfer the required volume of cell stock suspension to a centrifuge tube and centrifuge at an appropriate speed based on the specific animal species.
3. Resuspend the cells using your specific Hepatocyte Maintenance Medium (HMM), at the desired concentration for your experiment.

Note: For suspension culture experiments, use the cell suspension immediately or store it briefly at 4°.

4. The prepared cell suspension is now ready for short-term metabolic applications, such as drug uptake, metabolic stability, and metabolite profiling based on your study design.

In Vitro Hepatic Models
for ADME & Toxicology Studies in Drug Discovery

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PRODUCT ORDERING INFORMATION & TECHNICAL SUPPORT

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