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Suspension and Plateable Cryopreserved Hepatocytes

Single donor - technical information & instructions

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I. Introduction

This protocol is suitable for the thawing and culturing of suspension and plateable cryopreserved hepatocytes. Please read through this entire protocol before attempting this procedure. The health of the hepatocytes is dependent upon following the protocol carefully. For all cryopreserved hepatocytes, a thawing medium is required to ensure good viability following thaw; a plating medium is required to encourage hepatocyte attachment (for plated applications); and a serum-free maintenance medium is required to maintain healthy hepatocytes for the duration of your experiment. Primary hepatocytes are non-proliferative and cannot be passaged. Primary hepatocytes seeded at low density will de-differentiate and fail to replicate *in vivo* hepatocyte functions. Therefore, it's important to follow the counting and seeding guidelines to make sure your hepatocytes function properly for the duration of your experiments.

For answers to frequently asked questions and citations regarding these products, please visit our knowledge center:

https://knowledge.lonza.com

II. Required reagents and materials

(components sold separately)

- Cryopreserved hepatocytes
- Media (see Table 1 for appropriate media requirements by product and application)
- 37°C degree water bath
- Biological Safety Cabinet (BSC)
- Room temperature centrifuge capable of spinning 50 mL conical tubes at 65-200 x g
- 120 rpm orbital shaker inside a cell culture incubator (for suspension applications only)
- 37C/5% CO_2 Incubator

Consumables:

- Wide bore pipets and pipet tips
- Automated pipettor and serological pipet
- 0.4% solution of Trypan Blue
- Collagen coated cell culture plates (e.g. Corning™ BioCoat™ Collagen I Multiwell Plates) (for plated cells only)
- Overlay matrix (e.g. Corning® Matrigel® Matrix or equivalent), (for plated cells only)

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. Lonza guarantees the performance of these cells only if appropriate media and reagents are used exclusively and the recommended storage and use protocols are followed.

Any modifications made to the recommended cell systems, including the use of alternative media, reagents or protocols, will void cell and media performance guarantees. If you need assistance in selecting the appropriate media, reagents or protocol, please contact Lonza Scientific Support.

Table 1. Media requirements by species and application type

Species	Applicationt type	Thawing media (cat.no.)	Plating media (cat.no.)	Maintenance media (cat.no.)
Human	Single Donor Suspension	MCHT50		CC-3198
	Plated	MCHT50	MP100/MP250	CC-3198
Monkey	Suspension	MCRT50		CC-3198
	Plated	MCRT50	MP100/MP250	CC-3198
Dog	Suspension	MCAT50		CC-3198
	Plated	MCAT50	MP100/MP250	CC3198
Mouse	Suspension	MCRT50		MM250
	Plated	MCRT50	MP100/MP250	MM250
Rat	Suspension	MCRT50		CC-3198
	Plated	MCRT50	MP100/MP250	CC-3198

III. General Cell Information

Application	Species	Cat. no.	Description	Characterization (see website for more details)
Plated format	Human	HUCPG	Human Hepatocytes, Plateable Qualified	Viable and plateable for 5 days or greater in monolayer sandwich culture
		HUCPM	Human Hepatocytes, Metabolism Qualified	Viable and plateable for 3 days or greater in monolayer sandwich culture. Intrinsic clearance determined for CYP3A4, CYP2D6, and CYP2C9
		HUCPI	Human Hepatocytes, Induction Qualified	Viable and plateable for 5 days or greater in monolayer sandwich culture; mRNA and specific enzyme activity profiled following induction of CYP1A2, CYP3A4, and CYP2B6 enzymes
	Monkey	CYCP01	Monkey Hepatocytes Cyno, Plateable Qualified	Viable and plateable for 3 days in culture; Suspension ECOD analysis for general metabolism, SULT, and UGT activities measures using 7-ethoxycoumarin substrate and measurement of metabolites by mass spectrometry
		RHCP01	Monkey Hepatocytes Rhesus, Plateable Qualified	
	Dog	DBCP01	Dog Hepatocytes Beagle, Plateable Qualified	
	Mouse	MBCP01	Mouse Hepatocytes C57Bl/6, Plateable Qualified	
		MCCP01	Mouse Hepatocytes CD-1, Plateable Qualified	
		MXCP01	Mouse Hepatocytes B6C3F1, Plateable Qualified	
	Rat	RICP01	Rat Hepatocytes Wistar, Plateable Qualified	
		RSCP01	Rat Heptocytes SD, Plateable Qualified	
		RWCP01	Rat Hepatocytes WH, Plateable Qualified	
Suspension formats	Human	HUCSD	Human Hepatocytes, Suspension Qualified	Suspension ECOD analysis for general metabolism, SULT, and UGT activities measures using 7-ethoxycoumarin substrate and measurement of metabolites by mass spectrometry
	Monkey	CYCS01	Monkey Hepatocytes Cyno, Suspension Qualified	
		RHCS01	Monkey Hepatocytes Rhesus, Suspension Qualified	
	Dog	DBCS01	Dog Hepatocytes Beagle, Suspension Qualified	

Mouse	MBCS01	Mouse Hepatocytes C57BI/6, Suspension Qualified
	MCCS01	Mouse Hepatocytes CD-1, Suspension Qualified
	MXCS01	Mouse Hepatocytes B6C3F1, Suspension Qualified
Rat	RICS01	Rat Hepatocytes Wistar, Suspension Qualified
	RSCS01	Rat Hepatocytes SD, Suspension Qualified
	RWCS01	Rat Hepatocytes WH, Suspension Qualified

IV. Unpacking and Storage Instructions

1. **For cryopreserved cells**: Remove cryovials from the liquid nitrogen shipping dewar and immediately place into vapor phase liquid nitrogen storage. Alternatively, thaw and use the cells immediately. If shipping dewar is warm, please contact Customer Service.
2. **Maintenance medium for human, monkey, rat, and dog**. BulletKit™ Medium instructions: store basal medium (HBM™) at 2° - 8°C and SingleQuots™ Kit (HCM™) at ≤20°C in a freezer that is not self-defrosting. Once thawed, SingleQuots™ Kit should be stored at 2° - 8°C and added to basal medium within 72 hours. After SingleQuots™ Kit is added to basal medium, use within 1 month. **Do not re-freeze**.
3. **Maintenance medium for mouse:** unpack MM250-1 basal medium and MM250-2 supplement and store at 2° - 8°C. Once combined, use within 1 month.
4. **For plating medium (all species):** unpack MP100-1 (or MP250-1) basal medium and MP100-2 (or MP250-2) supplement and store at 2° - 8°C. Once combined, use within 1 month.
5. **For thawing medium (all species):** unpack the 50 mL conical tubes containing 40-45 mL of thawing medium and store upright at 2° - 8°C. Use by expiration date listed on the label.

V. Preparation of culture media

1. Decontaminate external surfaces of all vials and the medium bottle with ethanol or isopropanol.
2. To complete the hepatocyte plating media (MP100 and MP250), pour the entire contents of the vial labeled 'Plating Supplement' into the media.
3. **For Human, Monkey, Rat, and Dog**: to formulate the hepatocyte maintenance medium used for human, monkey, rat, and dog species, transfer the contents of the HCM™ SingleQuots™ Kit [Catalog No. CC-4182 containing Ascorbic Acid, Bovine Serum Albumin – Fatty Acid Free (BSA-FAF), Hydrocortisone, human Epidermal Growth Factor (hEGF), Transferrin, Insulin, and Gentamicin/Amphotericin-B (GA)] to HBM™ Basal Medium with a pipette, and rinse each vial with medium. Store at 4ºC for up to 1 month.
4. **For Mouse**: to complete the hepatocyte maintenance medium used for mouse hepatocytes, MM250, pour the entire contents of the supplement tube (MM250-2) into the basal medium (MM250-1). Store at 4ºC for up to 1 month.
5. When preparing these media, it may not be possible to recover the entire volume listed for each vial. Small losses (up to 10%) should not affect the cell growth characteristics of the supplemented medium.

NOTE: If there is concern that sterility was compromised during the supplementation process, the entire newly prepared culture medium may be re-filtered with a 0.2 µm filter to assure sterility. Routine re-filtration is not recommended.

VI. Thawing of cells

1. Warm media in a 37°C water bath as described below, depending on the experimental format needed. Refer to Table 1 for species specific thawing medium requirements.

   a. **Plated in Sandwich Model**: if an overlay of Matrigel or other basement membrane extract is to be used, warm thawing media and plating media only. Keep maintenance medium at 4 ºC.

   b. **Plated, but no overlay:** warm thawing media, plating media and maintenance medium to 37ºC.

   c. **Suspension**: warm the appropriate thawing and maintenance medium for your application. Once the thawing medium is warmed, disinfect it (70% ethanol wipe or spray) and transfer it to the BSC.

NOTE: The next 6 steps should occur as quickly as possible and without distraction

2. Remove the cryopreserved hepatocytes from their storage location (shipping dewar or storage dewar) and quickly submerge as much of the vial as possible, up to the cap, in the water bath. It is important to make sure the cap of the vial stays above the waterline.
3. Thaw the vial for approximately 90–120 seconds. The vial will thaw from the outside to the inside. You can see a spindle form and shrink as the vial thaws.
4. When almost completely thawed and only a small spindle of frozen cells remains, remove vial from water bath, disinfect the vial and transfer it to the BSC.
5. Quickly remove vial cap and carefully pour or pipette (with a wide-bore tip) hepatocytes into the 50 mL conical tube of appropriate warmed thawing medium.
6. Pipette approximately 1 mL thawing medium back into the original vial and pour or pipette the remaining cells back into the 50 mL tube of thawing medium to ensure that all hepatocytes are transferred.
7. Suspend the cells by carefully rocking the 50 mL tube by hand, for a few seconds. DO NOT VORTEX.
8. Centrifuge at room temperature, following the guidelines in Table 2.
9. Remove tube from centrifuge, disinfect, and transfer to the BSC.
10. Pour supernatant into a waste bottle, inverting completely, without shaking (or aspirate off supernatant carefully with a vacuum aspirator).
11. For each vial, gently resuspend cells in warm 3 mL plating medium or maintenance medium (for plated or suspension applications, respectively)
12. Determine the viability and yield of your hepatocytes using the Trypan Blue exclusion method (See section VII Cell Counting Procedure for assistance).

Table 2. Spin speed and duration

Species	Spin speed (g)	Duration (min
Single Donor Human	100	8
Monkey	100	8
Dog	65	4
Mouse	100	4
Rat	100	10

VII. Procedure for cell counting

To determine cell viability and viable cell yield with the Trypan Blue Exclusion Method for Hepatocytes, follow the directions below. Trypan Blue Exclusion Method must be used to accurately determine viability and yield of hepatocytes. Use of any other method may result in viability and yield different from that shown on the lot specific CofA.

1. To a clean microfuge tube, add 50 µL of 0.4% Trypan Blue Solution, 350 µL of Hepatocyte Plating media and 100 uL of cell suspension. Following this example results in a 1:5 fold dilution of your hepatocytes. If a different dilution is desired, volumes may be adjusted as long as the Trypan Blue still represents no more than 10% of the total volume.
2. Determine cell viability using the formula below.

   Eq. 1: 100 x (Live cell count ÷ Total cell count) = Viability%

3. Determine total viable cell yield using the formula below.

   Eq. 2: Viable cell count ÷ Quadrants counted x Dilution factor x 10000 x Current volume (mL) = Viable cell yield

   Example: 100 cells ÷ 4 quadrants x 5 x 10000 x 3mL total volume = 3,750,000 cells

VIII. Initiation of culture process

Procedure for suspension use

1. Add additional maintenance medium or your experimental buffer to bring cells to desired concentration of experimental design (most commonly, $1x10^6$ cells/mL).
2. It is recommended that you allow the hepatocytes to acclimate for 10 minutes by placing them on an orbital shaker located inside the incubator at 120 rpm. Your hepatocytes are now ready to use.

Procedure for plated use

1. Use the formulas below to determine the volume of plating medium to add to your current cell stock to achieve the desired cell density (refer to Table 3).

NOTE: Cell density is a key component of successful hepatocyte cultures. Recommended densities are provided as a range because small variances in counting and handling can results in different plating efficiencies. Therefore some optimization may be required by the end user to ensure optimal culture conditions for each individual lot.

Table 3 Desired cell density in plating medium by species and plate format (millions of cells/mL)

Species	6-well	12-well	24-well	48-well	96-well
Human	0.9-1.1	0.8-1.0	0.9-1.1	0.6-0.8	0.9-1.1
Monkey	1.0-1.4	0.9-1.4	0.9-1.4	0.8-1.1	1.0-1.4
Dog	0.9-1.1	0.8-1.0	0.9-1.1	0.6-0.8	0.9-1.1
Mouse	0.5-0.7	0.4-0.6	0.4-0.5	0.2-0.4	0.5-0.7
Rat	0.9-1.1	0.8-1.0	0.7-0.9	0.6-0.8	0.9-1.1

Eq. 3: Live cell yield (millions of cells) ÷ Desired cell density x (cells/mL) = Total volume needed (mL)

Eq. 4: Total volume needed (mL) - Current volume (mL) = Volume to add to cell stock (mL)

2. Using a 1 mL pipettor, gently transfer hepatocytes to a BioCoat Collagen I coated multi-well plate. Refer to Table 4 for correct volume of cell stock to add to each well and Table 5 for approximate cells/well.

NOTE: For 96-well plates, add 50 µL of plating media to each well followed by 50 µL of cell stock to uniformly disperse hepatocytes.

Table 4. Volume of plating medium with cells per well – all species

Plate format	6-well	12-well	24-well	48-well	96-well
Cell volume/well (mL/well)	2.0 mL/well	1.0 mL/well	0.50 mL/well	0.20 mL/well	0.05 mL blank media/ well + 0.05 mL 2X cells/well

Table 5. Approximate number of cells per well (in millions)

Species	6-well	12-well	24-well	48-well	96-well
Human	2.0	0.9	0.5	0.14	0.05
Monkey	2.4	1.1	0.5	0.18	0.06
Dog	2.0	0.9	0.5	0.14	0.05
Mouse	1.2	0.5	0.25	0.06	0.018
Rat	2	0.9	0.4	0.14	0.045

NOTE: We recommend you visually check seeding density to ensure a confluent monolayer.

3. Place plate in a 37°C/5% CO_2 incubator. It is very important that cells are evenly dispersed in the wells. This can be accomplished by moving plate in a north south east west motion, while maintaining contact with the incubator shelf. Failure to evenly disperse cells after placing in the incubator can result in accumulation of cells in the center of each well.

NOTE: This should not be attempted with 96-well plates. To avoid a vortex effect, with cells accumulating in the center of wells, leave 96-well plate undisturbed in BSC for 2-3 minutes before carefully placing in cell culture incubator. This will allow time for the cells to settle on the bottom of the wells and they will be less likely to congregate in the center.

4. Without removing plate from the incubator, repeat the shaking motion at 15, 30, and 45 minutes post-seeding. (Except 96-well plates)
5. At 60 minutes, remove plates from the incubator, carefully aspirate the medium, and replace with fresh plating medium using the volumes indicated in Table 4 (using 100 uL for 96-well plates).
6. Incubate the cells for a minimum of 4-6 hours post-seeding.
7. If using an overlay, proceed to the next section. If not, replace the medium with warm maintenance medium or application specific medium according to your experimental guidelines.
8. Replace maintenance medium daily following Table 6.

Table 6. Maintenance medium volume per well

	6-well	12-well	24-well	48-well	96-well
Volume per well for media changes	1.5 mL	0.75 mL	0.5 mL	0.2 mL	75 µL

Procedure for overlay

Matrigel is stored at -20ºC and must be thawed at 4ºC. It is best to thaw a frozen stock in the refrigerator the day prior to use.

Overlay matrix and the maintenance medium used for its dilution should be kept at or below 4°C. Keep everything on ice when preparing and while using the overlay.

1. Cool maintenance medium on ice.
2. Calculate the volume of maintenance medium needed to feed your plate(s), using volumes shown in Table 6.

Example: For 3 plates of 24-well format;

3 plates x 24 wells x 0.5 mL = 36 mL

NOTE: Always include approximately 10% extra volume to account for loss during pipetting.

3. Find the protein concentration of the overlay matrix on its specification sheet. Use the formula below to determine how much overlay matrix to add to maintenance medium. We recommend a final overlay matrix concentration of 0.3 mg/mL.

   Eq. 5: Volume of medium needed (mL) x 0.3 mg/mL ÷ Overlay matrix concentration (mg/mL) = Volume of overlay matrix needed (mL)

4. Add the calculated amount of overlay matrix to cold maintenance medium on ice.
5. Remove plate(s) from incubator, aspirate plating media from each well and replace with cold overlay solution, following the volume/well guidelines in Table 6.
6. Return plate to incubator and incubate for at least 2 hours before use. Replace maintenance medium daily with volumes shown in Table 6.

IX. Product warranty

Cultures have a finite lifespan *in vitro.*

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When placing an order or for Scientific Support, please refer to the product numbers and descriptions listed above. For a complete listing of all Primary Cell Products, refer to the Lonza website or the current Lonza catalog. To obtain a catalog, additional information or want to speak with Scientific Support, you may contact Lonza by web, e-mail, telephone or mail. (See Page 1 for details).

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