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Freshly Isolated Hepatocytes
Technical information & instructions

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I. Introduction

This protocol is suitable for handling freshly isolated hepatocytes that arrive in either a suspension or plated format. Please read through this entire protocol before attempting this procedure. The health of the hepatocytes is dependent upon following the protocol carefully.

NOTE: Lonza ships all hepatocytes, plated and in suspension, in a cold preservation medium at 4°C. This medium becomes cytotoxic at 6°C and is not suitable for hepatocyte culture. It is very important to aspirate this media quickly, and replace with warm recommended media, once removed from the cold packaging.

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 is 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)

- Fresh hepatocytes
- Media (See Table 1 for appropriate media requirements by product and application)

For cells arriving in suspension

- 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)
- 37ºC/5% CO_2 incubator
- Wide bore pipets and pipet tips
- 0.4% solution of Trypan Blue
- Rat tail type I collagen coated cell culture plates (e.g. Corning™ BioCoat™ Collagen I Multiwell Plates) (for plated cells only)
- Optional: Overlay matrix (e.g. Corning® Matrigel® Matrix or equivalent), (for plated cells only)

For cells arriving in plated formats

- Biological Safety Cabinet (BSC)
- 37ºC/5% CO_2 incubator
- Wide bore pipets and pipet tips
- Automated pipettor and serological pipets

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

Format	Plating medium	Culture medium (HCM™)
Suspension	MP100	CC-3198
Plated	N/A	CC-3198

III. General cell information

Species (Strain)	Format	Cat. # Plated cells without Matrigel overlay	Cat. # Plated cells with Matrigel overlay	Cat # Suspension cells (well format not applicable)
Human	6-well	HUF06	HUF06OL	HUFS1M
	12-well	HUF12	HUF12OL	
	24-well	HUF24	HUF24OL	
	48-well	HUF48	HUF48OL	
	96-well	HUF96	HUF96OL	
Dog	6-well	DBF06	DBF06OL	DBFS1M
	12-well	DBF12	DBF12OL	
	24-well	DBF24	DBF24OL	
	48-well	DBF48	DBF48OL	
	96-well	DBF96	DBF96OL	
Monkey Cynomolgous	6-well	CYF06	CYF06OL	CYFS1M
	12-well	CYF12	CYF12OL	
	24-well	CYF24	CYF24OL	
	48-well	CYF48	CYF48OL	
	96-well	CYF96	CYF96OL	
Rat (Sprague Dawley)	6-well	RSF06	RSF06OL	RSF1M
	12-well	RSF12	RSF12OL	
	24-well	RSF24	RSF24OL	
	48-well	RSF48	RSF48OL	
	96-well	RSF96	RSF96OL	
Rat (Wistar Hannover)	6-well	RWF06	RWF06OL	RWFS1M
	12-well	RWF12	RWF12OL	
	24-well	RWF24	RWF24OL	
	48-well	RWF48	RWF48OL	
	96-well	RWF96	RWF96OL	
Rat (Wistar)	6-well	RIF06	RIF06OL	RIFS1M
	12-well	RIF12	RIF12OL	
	24-well	RIF24	RIF24OL	
	48-well	RIF48	RIF48OL	
	96-well	RIF96	RIF96OL	
Mouse (CD-1)	6-well	MCF06	MCF06OL	MCFS1M
	12-well	MCF12	MCF12OL	
	24-well	MCF24	MCF24OL	
	48-well	MCF48	MCF48OL	
	96-well	MCF96	MCF96OL	
Mouse (C57BL/6)	6-well	MBF06	MBF06OL	MBFS1M
	12-well	MBF12	MBF12OL	
	24-well	MBF24	MBF24OL	
	48-well	MBF48	MBF48OL	
	96-well	MBF96	MBF96OL	

IV. 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. To formulate Hepatocyte Culture Medium (HCM™ Medium), 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.
4. When preparing these BulletKit™ 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.

V. Unpacking and culture instructions

NOTE: Lonza ships all hepatocytes, plated and in suspension, in a cold preservation medium at 4°C. This medium becomes cytotoxic at 6°C and is not suitable for hepatocyte culture. It is very important to aspirate this media quickly, and replace with warm HCM™ Medium, once removed from the cold packaging.

Handling freshly plated hepatocytes

1. Prior to removing plates from packaging, complete HCM™ Medium and warm to 37°C.
2. Remove plates from shipping container, place plate in BSC, and remove all packaging.

NOTE: If additional time is needed, remove a cold pack from the shipping container and place plate on top to maintain cold temperature before removing media.

3. Quickly and carefully aspirate all shipping medium from each well.
4. Quickly replace with the appropriate volume of warm HCM™ Medium according to Table 2.

Table 2. HCM™ Medium volume per well

	6-well	12-well	24-well	48-well	96-well
Volume per well for media changes	1.5mL	0.8mL	0.3mL	0.2mL	70µL

5. Place the plates in a 5% CO_2 incubator at 37°C.
6. Allow the hepatocytes to acclimate overnight (12-18 hours) prior to use.
7. Replace the HCM™ Medium daily using the volumes indicated in Table 2.
8. Adjust protocol as per experimental design.

Handling fresh hepatocytes in suspension

1. Prior to removing plates from packaging, complete MP100 Plating Medium (if plating the cells is required) or HCM™ Medium (if not plating) and warm to 37°C.
2. Remove tube of hepatocytes from packaging. Slowly and gently invert tube several times to homogenously suspend the cells. Maintain on ice until ready to aspirate.
3. Centrifuge hepatocytes at 4°C. For human hepatocytes, centrifuge at 100 x g for 8 min. For animal hepatocytes, centrifuge at 60 x g for 4 minutes.
4. Carefully aspirate the shipping medium off the pellet of cells. (If preferred, you may leave ~1mL to prevent disrupting the pellet).
5. Resuspend the hepatocyte pellet in warm HCM™ Medium (for suspension assays) or warm Plating Medium (to plate hepatocytes). Add 0.3 mL for every million cells you expect to recover, adjusting for experimental conditions with higher or lower concentrations of cells.

VI. Procedure for cell counting

To achieve accurate cell counts, it is recommended to use a manual Trypan Blue Exclusion Method. 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 50µL of 0.4% Trypan Blue Solution, 350µL of Hepatocyte

Plating media and 100uL 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 10% of the total volume.

2. Determine cell viability using the formula below.

   Eq. 2: 100 x (Live cell count ÷ Total cell count) = Viability%

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

   Eq. 3: Viable cell count ÷ Quadrants counted x Dilution factor x 10,000 x Current volume (mL) = Viable cell yield

   Example: 100 cells ÷ 4 quadrants x 5 x 10,000 x 3mL total volume = 3,750,000 cells

Using fresh hepatocytes in suspension

For suspension assays, adjust the cell density using HCM™ Medium to meet your experimental design (typically 0.5-2.0 x10^6 live cells/mL). Proceed with experimental protocols.

Plating fresh hepatocytes

For plated assays, use the tables below to adjust the live cell density using plating medium to meet your experimental design.

NOTE: For plating cells in 96-well plates, add 50µL of Plating Medium to each well followed by 50µL of 2X cell stock. This uniformly disperses hepatocytes across the plating surface.

Table 3 Desired cell densities by species and plate format

Species	6-well	12-well	24-well	48-well	96-well (2x)
		Cell density (x 10^6cells/mL)			
Human, Dog	0.9-1.1	0.8-1.0	0.9-1.1	0.6-0.8	0.9-1.1
Cyno	1.1-1.3	1.0-1.2	0.9-1.1	0.8-1.0	1.1-1.3
Mouse	0.5-0.7	0.4-0.6	0.3-0.5	0.2-0.4	0.3-0.4
Rat	0.9-1.1	0.8-1.0	0.7-0.9	0.6-0.8	0.9-1.1

1. Using a 1 mL pipettor, gently transfer hepatocytes to a BioCoat Collagen I coated multi-well plate. Refer to Table 4 below for correct volume of cell stock to add to each well.
2. Place plate in a 37°C/5% CO_2 incubator. For all plate formats except 96-well, disperse the cells by moving the plate, with your hand on top of it, parallel to the incubator shelf in a north-south, east-west motion.

NOTE: For 96-well plates, place directly in the incubator without shaking.

3. Without removing plate from the incubator, repeat the shaking motion at 15, 30, and 45 minutes post-seeding. (Except 96-well plates)
4. At 60 minutes, remove plate(s) from the incubator, carefully aspirate the medium, and replace with fresh plating medium using the volumes indicated in Table 4 (Using 100uL for 96-well).
5. Incubate the cells for a minimum total of 4-6 hours post-seeding.
6. If using an overlay, proceed to the next section.

   If not, replace the medium with warm HCM™ Medium or application specific medium according to your experimental guidelines.

Table 4. Cell volume per well – all species

Plate format	6-well	12-well	24-well	48-well	96-well
Cell volume/well (mL/well) at seeding	2.0 mL/well	1.0 mL/well	0.50 mL/well	0.20 mL/well	0.05mL blank media/ well + 0.05mL 2X cells/well
Cell volume per/well (mL/well) all other media changes	1.5 mL/well	0.8 mL/well	0.3 mL/well	0.2 mL/well	0.07mL/well

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 HCM™ 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 HCM™ Medium on ice.
2. Calculate the volume of HCM™ Medium needed to feed your cells(s), using volumes shown in Table 2.

   Example: For 3 plates of 24-well format;
   3 plates x 24 wells x 0.5mL = 36mL

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 HCM™ Medium. We recommend a final overlay matrix concentration of 0.3 mg/mL.

   Eq. 1 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 HCM™ Medium.
5. Remove plate(s) from incubator, aspirate plating media form each well and replace with cold overlay solution, following the volume/well guidelines in Table 4.
6. Incubate for at least 2 hours before use. Replace HCM™ Medium daily with volumes shown in Table 4.

VII. Product warranty

Cultures have a finite lifespan *in vitro.*

Lonza guarantees the performance of primary cells only if the appropriate primary cell 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.

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).

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* diagnostic procedures.

WARNING: LONZA PRIMARY CELL PRODUCTS CONTAIN HUMAN SOURCE MATERIAL, TREAT AS POTENTIALLY INFECTIOUS. Each donor is tested and found non-reactive by an FDA-approved method for the presence of both HIV-1 and HIV-2, hepatitis B virus and hepatitis C virus. Where donor testing is not possible, cell products are tested for the presence of viral nucleic acid from HIV-1, HIV-2, hepatitis B virus, and hepatitis C virus. Testing cannot offer complete assurance that HIV-1, HIV-2, hepatitis B virus, and hepatitis C virus are absent. 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.