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3D spheroid culture of Lonza mouse hepatocytes for use in toxicity assays

Instructions for use

Safety statements

These products are not for human or animal *in vivo* or diagnostic use, including use as a diluent or as an excipient.

These products are for research use *only.*

WARNING: The HCM™ SingleQuot® Supplement Pack (Lonza part no. CC-4812) contains a human plasma-derived protein which has been pasteurized prior to its use in the formulation in accordance with standard safety and quality procedures. Handle in accordance with good industrial hygiene and safety practice. If you require further information, please contact your site safety officer or Scientific Support.

Preparation of reagents

All work should be performed in a laminar flow hood. Decontaminate the external surfaces of all supplement vials and the medium bottles with 70% ethanol or isopropanol.

1. **Hepatocyte Culture Medium (HCM™ Medium)**
   - **a.** Following manufacturer instructions, combine the contents of the HCM™ SingleQuots® Kit (Lonza part no. CC-4182) to the HBM™ Basal Medium (Lonza part no. CC-3199) with a pipette, and rinse each supplement vial with medium. Store at 4°C for up to 1 month.
2. **Spheroid formation medium**
   - a. HCM™ Medium with 20% FBS (Avantor part no. 97068-085 or similar) and 25 mM HEPES buffer (Lonza part no. BEBP17–737E)
     - i. For example: Add 9 mL FBS and 1.125 mL 1 M HEPES buffer to 34.875 mL HCM™ Medium for a total of 45 mL of Spheroid Formation Medium.

Rodent Hepatocyte thawing, plating, and spheroid formation

Note: All work is to be performed in a laminar flow hood.

1. **For each strain of rodent hepatocyte used:** Warm a 50 mL tube of **Rodent and Monkey Cryopreserved Hepatocyte Thawing Medium** (Lonza part no. MCRT50) in a 37°C water bath.
2. Remove cryopreserved mouse hepatocytes from liquid nitrogen and quickly submerge as much of the vial as possible in a 37°C water bath.
   a. **Keep the cap above the water line.**
   b. Thaw for 90–120 seconds until only a small spindle of frozen cells remains.
3. Remove from the water bath, disinfect the vial with 70% ethanol, and transfer to a biosafety cabinet.
4. Quickly remove the vial cap and carefully pour or pipette (with a wide-bore tip) hepatocytes into the 50 mL centrifuge tube of warmed thawing medium.
5. Pipette 1 mL of thawing medium back into the original vial, then pour or pipette the remaining cells back into the 50 mL tube of thawing medium to ensure all hepatocytes have been transferred.
6. Suspend the cells by carefully rocking the 50 mL tube by hand for a few seconds. DO NOT VORTEX.
7. Centrifuge the cells for 4 min at 100xg and room temperature.
8. Remove tube from the centrifuge, disinfect with 70% ethanol, and return to the biosafety cabinet.
9. Carefully remove the supernatant.
10. For each vial, gently resuspend cells in 3 mL of pre-warmed **spheroid formation medium.**
11. Count cells using trypan blue and a hemocytometer using a 1:5 dilution factor

   a. In a 1 mL microcentrifuge tube, combine 100 µL of cell suspension with 50 µL of 0.4% Trypan Blue solution (Gibco part no. 17-942E or similar) and 350 µL of Spheroid Formation Medium.
   b. Other volumes and dilutions may be used as long as trypan blue represents no more than 10% of total volume
12. Bring volume of cells to 1 x 10^6 cells/mL.
13. Remove an appropriate volume of the 1 x 10^6 cells/mL cell suspension and dilute to 15,000 cells/mL using Spheroid Formation Medium.
   a. Example: To make 95 mL final volume of cell suspension for seeding cells, take 1.43 mL of 1 x 10^6 cell suspension and add to 93.6 mL of Spheroid Formation Medium.
14. Gently rock tube to suspend cells, then seed at 100 µL cell suspension / well in an ultra-low attachment 96-well plate, remembering to gently rock throughout to keep cells suspended.
   a. DO NOT VORTEX CELLS.
   b. Makes a final seeding density of 1,500 hepatocytes / well.
15. Fill an outer ring of wells around the cells with HCM™ Medium or 1X PBS to help control evaporation during spheroid formation.
16. Incubate cells at 5% CO_2 and 37°C. Leave undisturbed (no medium changes, etc.) until at least day 5.
17. Perform 50% medium changes on days 5 and 6, carefully pipetting out medium from the side of the wells (place pipette tips at 45° angle) so as not to pipette out the spheroids. Add fresh, pre-warmed **HCM™ Medium**.
18. Dosing spheroids for cytotoxicity assays occurs on day 7 (see next section).

Example use: Drug-induced liver injury toxicity panel using acetaminophen

19. On day 7, prepare **2X drug dosing medium** by dissolving target drug in **HCM™ Medium** at an appropriate concentration and titrating down the plate.
   a. Example: Using acetaminophen, create a stock solution in HCM™ Medium of 2X your desired highest dose (e.g., 50 mM for a desired highest dose of 25 mM). Titrate the 2X drug dosing medium in HCM™ Medium to produce 8 concentrations ranging from 0–50 mM.
20. Perform 50% medium changes with **2X drug dosing medium** on day 7.
   a. Note: This results in the final desired drug concentration, e.g., 25 mM.
21. Every 2–3 days thereafter up to 21 days (for a total of 28 days in culture), perform 50% media changes with **1X drug dosing medium.**
   a. **Example:** Using acetaminophen, create a stock solution in HCM™ Medium of your desired highest dose (e.g., 25 mM). Titrate the 1X drug dosing medium in HCM™ Medium to produce 8 concentrations ranging from 0–25 mM.
22. A variety of assessments can be utilized to assess spheroid health and functionality and generate EC50 values for the drug being tested.
   a. **Example:** Set up plates to be harvested at appropriate time points (e.g., 24–72 hours post-dosing for acute toxicity studies or 7 to 14 days post-dosing for chronic toxicity studies). Using Lonza's ViaLight® Plus BioAssay Kit, test cytotoxicity at each time point based on total cellular ATP to generate dose response curves and EC50 values.
   b. **Example**: Using an Incucyte® Live Cell Imaging System for live-cell imaging, Cytotox Green Dye can be added to the drug dosing medium according to manufacturer instructions and cells can be monitored continuously for cytotoxicity over several time points in culture.

Ordering information

Catalog no.	Description	Size
MBCP01	Cryopreserved C57BL/6 Mouse Hepatocytes, Plateable	≥ 5 million cells
MCCP01	Cryopreserved CD-1 Mouse Hepatocytes, Plateable	≥ 5 million cells
MACP01	Cryopreserved Balb/c Mouse Hepatocytes, Plateable	≥ 5 million cells
MXCP01	Cryopreserved B63CF1 Mouse Hepatocytes, Plateable	≥ 5 million cells
MCRT50	Rodent and Monkey Cryopreserved Hepatocyte Thawing Medium	50 mL
CC-3199	HBM Basal Medium	500 mL
CC-4182	HCM™ SingleQuots® Supplements	1 kit
CC-3198	HCM™ Hepatocyte Culture Medium BulletKit®	1 kit
BEBP17-737E	1M HEPES Buffer	100 mL
LT07-321	Lonza ViaLight® Plus BioAssay Kit	10,000 test kit
LT07-121		1,000 test kit
LT07-221		500 test kit