3D Hepatic Spheroid Formation Protocol

TECHNICAL BULLETIN

Purpose

This protocol describes the basic method and recommendations for generating and culturing primary human hepatocytes in a 96-well spheroid format.

Precautions

Observe universal precautions when handling human-derived tissues and cells as they are potentially biohazardous. Refer to the guidelines set forth in Occupational Safety and Health Standards for handling blood, tissues, body fluids, or other potentially infectious materials. Follow institutional guidelines for the collection and disposal of all solid and liquid waste that has been in contact with these products.

Materials and Methods

Medium Preparation

1. One day before use, transfer Human Hepatocyte Plating Medium Supplement (HHPMS) from -20°C to 4°C storage and thaw overnight. **OPTIONAL:** Thaw at room temperature or 37°C prior to use.
2. Spray and wipe all reagent bottles (Human Hepatocyte Plating Medium (HHPM), Human Hepatocyte Plating Medium Supplement (HHPMS), and Human Hepatocyte Thawing Medium (HHTM)) with 70% (v/v) alcohol and place in Biological Safety Cabinet (BSC).
3. Prepare complete HHPM by adding 15 mL HHPMS to 250 mL HHPM. **NOTE:** If desired, an antibiotic can be added to complete HHPM. Penicillin/Streptomycin, at a final concentration of 50 units/mL and 50 µg/mL, respectively, is recommended.
4. Invert HHTM bottle to evenly mix, then filter HHTM through 0.2 µm filter. Transfer filtered HHTM to a sterile 50 mL conical tube. Cap 50 mL tube and gently invert 3 times to mix thoroughly.
5. Prior to use, warm reagents in 37°C water bath for 20-30 minutes.

Thawing and Plating of Primary Human Hepatocytes (PHHs)

1. Remove 50 mL conical tube of HHTM from 37°C water bath, spray, and wipe with 70% (v/v) alcohol, and place in BSC.
2. Gently invert the tube 3 times to mix HHTM thoroughly.
3. Refill portable LN_2 container with enough LN_2 to submerge a cryovial to at least half of its height.
4. Place small, insulated laboratory ice tray filled with ice, for transporting cells, next to 37°C water bath.
5. Quickly remove cryovial of human hepatocytes from LN_2 storage and place in the portable LN_2 container.
6. Carefully transport frozen cryovial in the LN_2 container to the 37°C water bath.
7. Using forceps or tongs, QUICKLY remove cryovial of human hepatocytes from the LN_2, loosen the cryovial cap slightly to release pressure and then re-tighten. **NOTE:** Do NOT completely remove the cryovial cap.
8. Partially submerge cryovial vertically in 37°C water bath to thaw for 90 seconds. Ensure the water level is at least even with the top of the frozen cell suspension, being careful to keep the neck of the cryovial above the water.
9. Remove cryovial from 37°C water bath and invert. The cell suspension should be somewhat liquefied and slide freely without tapping or shaking the vial. If frozen cell suspension does not slide freely, return cryovial to upright position and place back in water bath for an additional 5 seconds. Recheck the movement of the cell suspension. **CAUTION:** Do not over thaw. Thawing longer than necessary can reduce viability.
10. Quickly place cryovial in ice tray to prevent the cells from reaching room temperature, and transport to BSC. Wipe the cryovial with 70% (v/v) alcohol prior to placing in BSC.
11. Remove the cryovial cap and pour hepatocytes into tube containing HHTM. Using a 1000 µL pipette, rinse the cryovial once with approximately 1000 µL of HHTM from the tube. Transfer the HHTM back to the tube.
12. Tighten the tube cap and gently invert the tube 3 times to uniformly resuspend the hepatocytes.
13. Centrifuge the hepatocytes at 100 x g for 8 minutes at room temperature.
14. Return to BSC and gently aspirate the supernatant without disturbing the cell pellet in the bottom of the tube.
15. Add 5 mL of warm complete HHPM to hepatocytes. Gently rock the tube to resuspend the hepatocytes. **NOTE:** Do NOT pipette or vortex to mix or swirl vigorously.
16. Add 3 mL of additional warm complete HHPM to hepatocytes and count the suspension by either trypan blue exclusion method on a hemocytometer and/or AO/PI staining with an automated cell counter to determine yield and viability. **NOTE:** Use a 10-fold dilution for the trypan blue exclusion method or a 2-fold dilution for the AO/PI staining.
17. Add additional warm complete HHPM to cell suspension to dilute hepatocytes to the optimal seeding densities of 15,000 or 30,000 cells/mL.
18. Swirl or invert the tube of hepatocytes to ensure the cell suspension is homogenously mixed, then pour the cell suspension into a sterile reagent reservoir until half full.
19. Gently rock/shake the reagent reservoir in a N-S,E-W motion, being careful not to spill the PHH suspension throughout the seeding process to ensure the PHH suspension is well mixed.
20. Using a multi-channel pipette, transfer 100 µL of cell suspension from the reservoir to each well of the culture plate. If using 15,000 cells/mL, the final concentration per well should be 1,500 cells/well. If using 30,000 cells/mL, the final concentration per well should be 3,000 cells/well.
21. Add 100 µL warm complete HHPM to outer wells and any additional wells that are not being used in the 96-well plate.
22. Centrifuge the 96-well plate using plate adaptors at 100 x g for 2 minutes at room temperature.
23. Place the 96-well plate in 37°C incubator.
24. Do not change medium for wells until day 5 to allow the cells to form spheroids.

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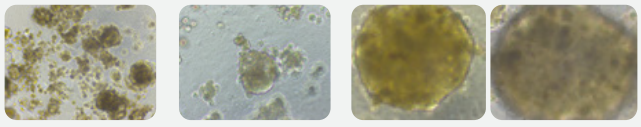
Thawing and Plating of Primary Human Hepatocytes (PHHs)

1. Visually inspect spheroid formation daily.
2. On day 4, transfer Human Hepatocyte Culture Medium Supplement (HHCMS) from -20°C to 4°C storage and thaw overnight. **OPTIONAL:** Thaw at room temperature or 37°C prior to use.
3. On day 5, prepare complete Human Hepatocyte Culture Medium (HHCM).
4. Spray and wipe all reagent bottles (HHCM and HHCMS) with 70% (v/v) alcohol and place in Biological Safety Cabinet (BSC).
5. Prepare complete HHCM by adding 5 mL HHCMS to 500 mL HHCM. **NOTE:** If desired, an antibiotic can be added to complete HHCM. Penicillin/Streptomycin, at a final concentration of 50 units/mL and 50 µg/mL, respectively, is recommended.
6. Mix well by gently inverting the capped container several times.
7. Filter sterilize the complete medium by using a 0.2 µm filter.
8. Prepare an aliquot of complete HHCM for media change and place in 37°C water bath 20-30 minutes prior to use. **NOTE:** Recommended volume of complete HHCM for one PHH lot is 50 mL.
9. Using a multi-channel pipet, gently replace medium with half volume (~50 µL) complete HHCM for each well, being careful not to disturb the spheroid.
10. Subsequent half volume medium changes should take place every other day (i.e. Monday, Wednesday, and Friday).
11. The recommended culture time for PHH spheroids is 21 days. See Figure 1 for representative images of ideal spheroid formation.



Individual aggregates forming but failing to coalesce into a single spheroid.

Aggregates forming, but border is not smooth. Spheroid is dark in color.

Good, compact, translucent spheroid, with clearly defined border.

Figure 1. Images for spheroid formation.

Materials

1. Consumables
   a. 70% (v/v) alcohol (ethanol or isopropyl alcohol)
   b. 70% alcohol wipes (or lab wipes soaked in 70% (v/v) alcohol)
   c. Laboratory wipes
   d. Ice
   e. Liquid nitrogen (LN_2)
   f. Sterile 50 mL conical centrifuge tubes
   g. Sterile 50 mL or 100 mL reagent reservoir (optional)
   h. LifeNet Health Cryopreserved Human Hepatocytes
   i. LifeNet Health Human Hepatocyte Thawing Medium (HHTM)
   j. LifeNet Health Human Hepatocyte Plating Medium (HHPM)
   k. LifeNet Health Human Hepatocyte Plating Medium Supplement (HHPMS)
   l. LifeNet Health Human Hepatocyte Culture Medium (HHCM)
   m. LifeNet Health Human Hepatocyte Culture Medium Supplement (HHCMS)
   n. Antibiotic (optional: product suggestion- Penicillin/Streptomycin)
   o. Either Trypan blue solution or AO/PI (acridine orange/propidium iodide) stain

1. Small Equipment
   a. Appropriate personal protective equipment (PPE)
   b. Laboratory ice tray capable of containing a small amount of liquid nitrogen and a cryovial box
   c. Hemocytometer or other cell-counting device
   d. Forceps or tongs
   e. Cryovial storage box
   f. Timer
   g. Pipettes (20, 200 and 1000 µL) with sterile tips
   h. Serological pipettor and sterile pipettes (1, 2, 5, 10, 25 mL)
   i. 1000 µL multichannel electronic pipette with sterile tips (optional)
   j. Ultra-low adhesion, U-bottom 96-well plates with lid (Corning cat. #4515) or NunclonTM SpheraTM 96-well U-Shaped-Bottom Microplate (ThermoFisher Scientific cat. #174925 or 174929
   k. Sterile disposable or washable media bottles (100-250 mL)
   l. 0.2 µm Polyethersulfone (PES) filters

1. Large Equipment
   a. Biological Safety Cabinet (BSC)
   b. Cryogenic storage freezer capable of temperatures ≤-150°C
   c. Portable liquid nitrogen (LN_2) dewar or other suitable container to transport frozen vials
   d. 37°C/5% CO_2 Incubator (humidified)
   e. 37°C water bath
   f. Centrifuge capable of achieving up to 200 g (96-well plate adaptors)
   g. -20°C freezer
   h. 4°C refrigerator
   i. Vacuum aspiration system with sterile aspiration tips (optional)

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