TECHNICAL MANUAL

Initiation, Growth, and Differentiation of Human Hepatic Organoids Using HepatiCult™

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1.0 Introduction

Liver organoids are miniature three-dimensional (3D) cell culture systems that serve as a valuable model for studying liver cell biology. They retain many features of in vivo hepatocytes and can recapitulate donor heterogeneity. They serve as a proliferative and physiologically relevant alternative to conventional two-dimensional (2D) cell culture for screening and the study of hepatic development, regeneration, detoxification, metabolism, and disease. We have developed the HepatiCult™ Organoid Kit (Human) and associated protocols as a novel cell culture system to support a complete hepatic organoid culture workflow. This includes robust organoid establishment from fresh or cryopreserved normal liver tissue in complete HepatiCult™ Organoid Initiation Medium (OIM), long-term expansion and scale-up of established organoids in complete HepatiCult™ Organoid Growth Medium (OGM), and differentiation of expanded organoids in complete HepatiCult™ Organoid Differentiation Medium (ODM) to generate matured organoids that exhibit some in vivo hepatocyte functionality. Organoids cultured in complete HepatiCult™ OGM can be adapted to a range of culture protocols including 2D monolayer cultures, dilute Corning® Matrigel® suspension cultures, and high-throughput-assay compatible culture formats. They also remain viable following cryopreservation and retain their capacity for propagation and maturation, thereby offering flexibility and convenience when working with tissue-derived samples.

Various kits are available to support partial and complete hepatic organoid workflows using either primary tissue or established organoids as the starting material, as outlined below.

- To support the complete hepatic organoid workflow, including organoid initiation, scale-up, and differentiation from primary tissue:
    - HepatiCult™ Organoid Kit (Human) (Catalog #100-0386)
- To initiate organoids from primary tissue:
    - HepatiCult™ Organoid Initiation Medium (Human) (Catalog #100-0384)
- To expand and scale-up established organoids (including cryopreserved organoids):
    - HepatiCult™ Organoid Growth Medium (Human) (Catalog #100-0385)
- To differentiate established organoids:
    - HepatiCult™ Organoid Growth Medium (Human) (Catalog #100-0385)
    - HepatiCult™ Organoid Differentiation Medium (Human) (Catalog #100-0383)

2.0 HepatiCult™ Organoid Initiation, Growth, and Differentiation Media

Media for the complete HepatiCult™ organoid workflow are available as a kit (HepatiCult™ Organoid Kit, Catalog #100-0386) or individually for each stage (initiation, growth, and differentiation) as outlined in the table below.

Refer to the Product Information Sheet (PIS; Document #10000008301) for component storage and stability information, available at www.stemcell.com or contact us to request a copy.

PRODUCT NAME	CATALOG #	COMPONENT NAME	COMPONENT #	QUANTITY
HepatiCult™ Organoid Kit (Human)	100-0386	HepatiCult™ Organoid Basal Medium (Human)	100-0387	3 x 95 mL
		Organoid Supplement	100-0191	50 mL
		HepatiCult™ Organoid Growth Supplement (Human)*	100-0389	2 x 5 mL
		HepatiCult™ Organoid Differentiation Supplement (Human)*	100-0388	5 mL
HepatiCult™ Organoid Initiation Medium (Human)	100-0384	HepatiCult™ Organoid Basal Medium (Human)	100-0387	95 mL
		Organoid Supplement	100-0191	50 mL
		HepatiCult™ Organoid Growth Supplement (Human)*	100-0389	5 mL
HepatiCult™ Organoid Growth Medium (Human)	100-0385	HepatiCult™ Organoid Basal Medium (Human)	100-0387	95 mL
		HepatiCult™ Organoid Growth Supplement (Human)*	100-0389	5 mL
HepatiCult™ Organoid Differentiation Medium (Human)	100-0383	HepatiCult™ Organoid Basal Medium (Human)	100-0387	95 mL
		HepatiCult™ Organoid Differentiation Supplement (Human)*	100-0388	5 mL

*This product contains material derived from human plasma. Donors have been tested and found negative for hepatitis B surface antigen (HBsAg) and HIV-1 antibodies and/or HIV-1 antigen. However, this product should be considered potentially infectious and treated in accordance with universal handling precautions.

3.0 Preparation of Media

Prepare the following as directed in the protocols for organoid initiation (section 4.0), growth (section 5.0), and differentiation (section 6.0).

3.1 Complete HepatiCult™ Organoid Initiation Medium (OIM)

Use sterile technique to prepare complete HepatiCult™ OIM (HepatiCult™ Organoid Basal Medium + Organoid Supplement + HepatiCult™ Organoid Growth Supplement + Y-27632 + antibiotics). The following example is for 100 mL of complete medium. If preparing other volumes, adjust accordingly.

1. Thaw HepatiCult™ Organoid Growth Supplement and Organoid Supplement overnight at 2 - 8°C. Mix thoroughly.

   Note: If not used immediately, aliquot and store at -20°C. Do not exceed the shelf life of the components. After thawing aliquots, use immediately. Do not re-freeze.

2. Combine the following:
   - 5 mL HepatiCult™ Organoid Growth Supplement
   - 50 mL Organoid Supplement
   - 44.8 mL HepatiCult™ Organoid Basal Medium
   - 200 µL of 5 mM Y-27632 in water (final concentration 10 µM)
   - Antibiotics (e.g. final concentration 50 µg/mL gentamicin)
3. Mix thoroughly. Warm to room temperature (15 - 25°C) before use.

   Note: If not used immediately, store complete HepatiCult™ OIM at 2 - 8°C for up to 2 weeks.

3.2 Complete HepatiCult™ Organoid Growth Medium (OGM)

Use sterile technique to prepare complete HepatiCult™ OGM (HepatiCult™ Organoid Basal Medium + HepatiCult™ Organoid Growth Supplement + antibiotics). The following example is for preparing 100 mL of complete medium. If preparing other volumes, adjust accordingly.

1. Thaw HepatiCult™ Organoid Growth Supplement overnight at 2 - 8°C. Mix thoroughly.

   Note: If not used immediately, aliquot and store at -20°C. Do not exceed the shelf life of the supplement. After thawing aliquots, use immediately. Do not re-freeze.

2. Add 5 mL of Growth Supplement to 95 mL of Basal Medium. Add antibiotics (e.g. final concentration 50 µg/mL gentamicin). Mix thoroughly. Warm to room temperature (15 - 25°C) before use.

   Note: If not used immediately, store complete HepatiCult™ OGM at 2 - 8°C for up to 2 weeks.

3.3 Complete HepatiCult™ Organoid Differentiation Medium (ODM)

Use sterile technique to prepare complete HepatiCult™ ODM (HepatiCult™ Organoid Basal Medium + HepatiCult™ Organoid Differentiation Supplement + antibiotics + dexamethasone). The following example is for preparing 100 mL of complete medium. If preparing other volumes, adjust accordingly.

1. Thaw HepatiCult™ Organoid Differentiation Supplement overnight at 2 - 8°C. Mix thoroughly.

   Note: This supplement is light sensitive; minimize exposure to light.

   Note: If not used immediately, aliquot and store at -20°C. Do not exceed the shelf life of the supplement. After thawing aliquots, use immediately. Do not re-freeze.

2. Combine the following:
   - 5 mL HepatiCult™ Organoid Differentiation Supplement
   - 94.9 mL HepatiCult™ Organoid Basal Medium
   - Antibiotics (e.g. final concentration 50 µg/mL gentamicin)
3. Mix thoroughly.

   Note: If not used immediately, store complete HepatiCult™ ODM at 2 - 8°C for up to 2 weeks. On days when the medium is required, aliquot the required volume in a tube and warm to room temperature before use. HepatiCult™ ODM is light sensitive; minimize exposure to light.
4. Immediately before use, aliquot the volume of complete HepatiCult™ ODM required to perform a full-medium change and add 1 µL of 3 mM dexamethasone in dimethylsulfoxide per mL of complete HepatiCult™ ODM (final concentration 3 µM). Mix thoroughly.

   Note: Leftover volumes of complete HepatiCult™ ODM containing dexamethasone should be discarded.

4.0 Initiation of Hepatic Organoids

The following protocol is for establishing human hepatic organoids from up to 1 g of human liver tissue in Corning® Matrigel® domes. For other tissue amounts, adjust volumes accordingly. Fresh or frozen liver tissue can be used; if using fresh tissue, process within 48 hours.

4.1 Materials Required

PRODUCT	CATALOG #
24-well tissue culture-treated plate	38017
HepatiCult™ Organoid Initiation Medium (Human)	100-0384
Y-27632	72302
Antibiotics (e.g. gentamicin)	---
Ammonium Chloride Solution	07800
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free, LDEV-free (≥ 8 mg/mL protein)	Corning 356231
Bovine serum albumin (BSA)	---
Collagenase Type IV	07427
DNase I Solution (1 mg/mL)	07900
DMEM/F-12 with 15 mM HEPES	36254
Fetal bovine serum (FBS)	---
Advanced DMEM/F-12	Thermo Fisher 12634028
TrypLE™ Express Enzyme, no phenol red	Thermo Fisher 12604039
Trypan Blue	07050
Culture Dish, Non-Treated, 100 mm	38045
D-PBS (Without Ca++ and Mg++)	37350
10 mL and 25 mL serological pipettes	e.g. 38004 and 38005
15 mL and 50 mL conical tubes	e.g. 38009 and 38010

4.2 Equipment Required

- Biohazard safety cabinet certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- Pipettors (e.g. Catalog #38058) with appropriate tips
- Inverted microscope
- Refrigerator (2 - 8°C)
- Styrofoam box with ice
- Water bath at 37°C
- Dissection tools

4.3 Setup and Reagent Preparation

1. Place a 24-well tissue culture-treated plate in a 37°C incubator for at least 1 hour.
2. Thaw ~40 µL of Corning® Matrigel® on ice for each dome to be seeded. Keep Matrigel® on ice when handling to prevent it from solidifying.
3. Place Ammonium Chloride Solution and a 50 mL conical tube on ice.
4. Prepare complete HepatiCult™ OIM (section 3.1).

   Note: ~4.5 mL of complete HepatiCult™ OIM is required for organoid initiation in each dome. The volume of OIM supplied in one kit is sufficient to initiate organoids in ~22 wells of a 24-well plate.
5. Prepare the following reagents:

   a. Wash Solution (70 mL)

   Combine the following:

   - 0.7 mL fetal bovine serum (FBS)
   - 69.3 mL DMEM/F-12 with 15 mM HEPES

   Mix thoroughly and store on ice. Add 15 mL of cold (2 - 8°C) Wash Solution to a 100 mm dish and place on ice.

   b. Tissue Dissociation Cocktail (25 mL)

   Combine the following:

   - 62.5 mg Collagenase Type IV
   - 250 µL DNase I Solution (1 mg/mL)
   - 24.75 mL DMEM/F-12 with 15 mM HEPES

   Mix thoroughly. Store at 2 - 8°C for up to 2 weeks. Warm to 37°C before use.

   c. AdvDMEM + DNase I (26 mL)

   Combine the following:

   - 25 mL Advanced DMEM/F-12
   - 1 mL of 25% bovine serum albumin (BSA) in water
   - 150 µL of DNase I Solution (1 mg/mL)
   - 50 µL of 5 mM Y-27632 (final concentration 10 µM)

   Mix thoroughly. Store on ice.

   d. TrypLE™ + BSA (10 mL)

   Combine the following:

   - 10 mL TrypLE™ Express
   - 400 µL 25% BSA in water

   Mix thoroughly. Store on ice.
6. Optional: Hepatic ducts may adhere to the surfaces of conical tubes and serological pipettes. To simplify handling and minimize this adherence, pre-wet conical tubes and serological pipettes with Anti-Adherence Rinsing Solution (Catalog #07010) and Advanced DMEM/F-12, as follows:
   a. Prepare 25 mL aliquots of Anti-Adherence Rinsing Solution and Advanced DMEM/F-12 in conical tubes. Keep aliquots at room temperature (15 - 25°C).
   b. Coat serological pipettes with aliquoted Rinsing Solution followed by Advanced DMEM/F-12 immediately before use.

c. Transfer 5 mL of aliquoted Rinsing Solution to a 15 mL conical tube (or 10 mL of Rinsing Solution to a 50 mL conical tube). Swirl to coat the tube. Transfer the Rinsing Solution to additional tubes as required, swirling to coat each one.
d. Aspirate Rinsing Solution from tubes. Repeat steps c and d with Advanced DMEM/F-12, then aspirate Advanced DMEM/F-12.
e. Cap all coated tubes and store at room temperature until required.

4.4 Initiation Protocol

Complete the setup and reagent preparation as described in section 4.3 before beginning the protocol below.

4.4.1 Tissue Preparation

1. Weigh human liver tissue specimen. Under aseptic conditions, transfer tissue to the dish containing 15 mL cold Wash Solution (prepared in section 4.3).
2. Use scissors to cut tissue into small (~3 - 5 mm) pieces.
3. Using a 25 mL serological pipette, transfer liver pieces and Wash Solution into a 50 mL conical tube on ice. Pipette the entire suspension up and down 5 times using the same 25 mL serological pipette.

   Note: Larger tissue pieces can be aspirated by tapping the opening of the pipette against the surface of the Petri dish while aspirating.
4. Let liver pieces settle by gravity on ice for 1 - 2 minutes. Aspirate and discard supernatant.
5. Add 10 mL of cold Wash Solution to the liver pieces. Using a 10 mL serological pipette, **vigorously** pipette the entire volume up and down 10 times with medium-high force.

   Note: The opening of the serological pipette can be tapped against the bottom of the tube to help with aspiration of larger tissue pieces.
6. Let liver pieces settle by gravity for 1 minute. Using a 10 mL serological pipette, remove and discard the supernatant.
7. Add 5 mL warm Tissue Dissociation Cocktail to liver pieces. Using a 10 mL serological pipette, **vigorously** pipette the entire volume up and down 10 times with medium-high force.

   Note: The opening of the serological pipette can be tapped against the bottom of the tube to help with aspiration of larger tissue pieces.
8. Incubate tube in a 37°C water bath for 15 minutes.
9. Remove tube from the water bath. Using a 10 mL serological pipette, **vigorously** pipette the liver pieces up and down 10 times with medium-high force.
10. Let liver pieces settle by gravity for 1 minute. Using a 10 mL serological pipette, collect the supernatant and add to the 50 mL conical tube on ice. Keep this tube on ice.
11. Repeat the digestion cycle in steps 7 - 10, pooling supernatants in the 50 mL tube on ice. Continue repeating these steps until tissue pieces have been completely dissociated into hepatic ducts and no tissue pieces remain.

    Note: The enzymatic tissue dissociation usually requires 4 x 15-minute digestion cycles, but a fifth digestion cycle can be carried out if tissue pieces still remain. The pooled supernatant volume will be ~20 - 25 mL.
12. Centrifuge the tube containing pooled supernatants at 290 x *g* for 5 minutes. Aspirate and discard as much of the supernatant as possible without disturbing the pellet.
13. Add 10 mL TrypLE™ + BSA to the pellet and pipette vigorously to resuspend.

14. Incubate tube in a 37°C water bath for 10 minutes. Remove tube from water bath, vigorously resuspend ductal material again using a 10 mL serological pipette, and return to the 37°C water bath for an additional 10 minutes.
15. Remove tube from water bath and pipette vigorously using a 10 mL serological pipette to resuspend. Add 10 mL AdvDMEM + DNase I to the tube.

    Optional: A filtration step using a 100 µm reversible cell strainer can be used to separate the larger ductal material from smaller ductal fragments. The ductal fragments are easier to manipulate and will yield cleaner but less dense cultures. The ductal material captured on the cell strainer can also be used to initiate organoid cultures. However, this material is sticky and can be challenging to manipulate or dissociate further.
16. Add 25 - 30 mL of cold Wash Solution to the tube to bring the total volume up to 50 mL.
17. Centrifuge at 290 x *g* for 10 minutes. Carefully aspirate and discard as much of the supernatant as possible without disturbing the pellet.
18. Add 2 mL of cold AdvDMEM + DNase I to the pellet and pipette up and down to resuspend.
19. Add 8 mL cold Ammonium Chloride Solution and mix thoroughly. Leave the tube on ice for 5 minutes to lyse any red blood cells.
20. Centrifuge at 290 x *g* for 10 minutes. Aspirate as much of the supernatant as possible without disturbing the pellet.
21. Add 15 mL cold Wash Solution to the pellet and resuspend thoroughly. Transfer the entire volume to a 15 mL conical tube.
22. Centrifuge at 290 x *g* for 10 minutes. Aspirate as much of the supernatant as possible without disturbing the pellet.
23. Add 1 mL cold AdvDMEM + DNase I to resuspend the pellet. Perform a live cell count using Trypan Blue and a hemocytometer.

4.4.2 Dome Culture Initiation

1. Add 10 mL cold AdvDMEM + DNase I to the suspension from section 4.4.1 step 23. Pipette up and down 3 - 5 times to create a uniform suspension. Immediately transfer equal volumes into at least 2 and up to 24 new 15 mL tubes.

   Note: Each 15 mL tube will be used to seed one Matrigel® dome. The number of domes that can be seeded will depend on the size of the original tissue sample and the live cell count. At least 20,000 live cells should be seeded per dome, but higher seeding densities are recommended if possible.
2. Centrifuge the tubes at 290 x *g* for 5 minutes. Aspirate as much of the supernatant as possible without disturbing the pellet, leaving 5 - 10 µL in the tube (the pellet is often not visible). Place the tube on ice.
3. Remove the 24-well plate from the incubator and place in a biosafety cabinet.
4. Process one tube/pellet at a time, as described below. Work quickly to ensure the Matrigel® does not solidify. Pipette tips can be cooled prior to working with Matrigel® to help minimize premature solidifying.

   Note: The 8 wells in the center of a 24-well plate are the most suitable for domes, since their surfaces are the most even. Wells at the edges of the plate are often slightly slanted, contributing to domes touching the wall of the well and flattening out.

   a. Using a pipettor with a 200 µL pipette tip, add 30 µL of thawed Matrigel® on top of the pellet. Without generating bubbles, gently mix the duct-Matrigel® suspension by pipetting up and down 5 - 8 times, going only to the first stop of the pipettor.
   b. Set the pipettor volume to 40 µL. Add the entire suspension to the center of one well of the 24-well plate to form a dome. While dispensing, gradually move the pipette tip upward so that the cells are

evenly distributed throughout the dome. Dispense only to the first stop of the pipettor to avoid generating bubbles on top of the dome.

5. Repeat step 4 for the remaining pellets/tubes.
6. Place the lid on the culture plate. Carefully place the plate in an incubator at 37°C and 5% CO_2 for 10 minutes to let the domes solidify.
7. Remove the plate from the incubator and place in the biosafety cabinet. Without disturbing the domes, carefully add 750 µL of room temperature (15 - 25°C) complete HepatiCult™ OIM against the side of each well containing a dome. Do not pipette directly onto the domes.
8. Add sterile D-PBS to any unused wells. Place the lid on the culture plate. Incubate at 37°C and 5% CO_2.
9. Perform a full-medium change every 2 - 3 days by carefully aspirating the medium and adding 750 µL of fresh complete HepatiCult™ OIM at room temperature.

   Note: If Matrigel® domes are loose, remove 500 µL of medium from the well, then add 500 µL of fresh medium. To avoid weekend medium changes, perform medium changes on Mondays, Wednesdays, and Fridays.

   Note: To monitor organoid growth, take photos of the same field of view every 2 - 3 days until they are passaged.
10. Passage organoids before the lumen turns dark and the organoids collapse (up to 2 weeks), as described in section 5.0. Refer to Figure 1 for representative images.

    Note: Organoid morphologies during the 2-week initiation period can vary and may not always resemble the morphologies shown in Figure 1. If initiated cultures appear sparse or only comprise small and dense-looking organoids at day 14, it is recommended to passage cultures using a 1:1 split ratio, as follows:

    - *Follow section 5.4 steps 1 - 4.*
    - *Transfer the entire volume of fragment suspension to a 15 mL conical tube containing 1 mL cold AdvDMEM + BSA.*
    - *Proceed to section 5.4 step 7.*

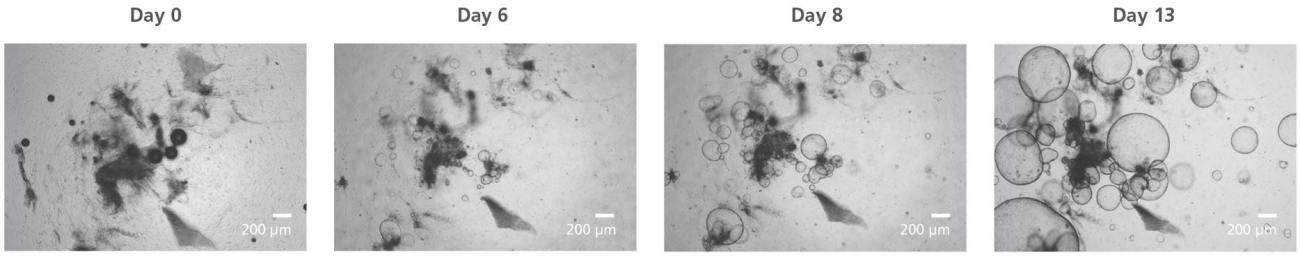


Figure 1. Establishment of Human Hepatic Organoids from Primary Hepatic Ducts in Complete HepatiCult™ OIM. Organoids are ready to passage when they resemble the organoids shown on Day 13. Magnification: 2X.

5.0 Growth of Hepatic Organoids

The following protocol is for passaging human hepatic organoids in dome cultures (established in section 4.0 or from cryopreserved sources) in 24-well plates. For passaging to a 96-well plate, refer to the appendix (section 7.0).

5.1 Materials Required

PRODUCT	CATALOG #
HepatiCult™ Organoid Growth Medium (Human)	100-0385
Antibiotics (e.g. gentamicin)	---
24-well tissue culture-treated plate	38017
6-well tissue culture-treated plate	38016
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free, LDEV-free (≥ 8 mg/mL protein)	Corning 356231
Bovine serum albumin (BSA)	---
Advanced DMEM/F-12	Thermo Fisher 12634028

5.2 Equipment Required

- Biohazard safety cabinet certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- Pipettors (e.g. Catalog #38058) with appropriate tips
- Inverted microscope
- Refrigerator (2 - 8°C)
- Styrofoam box with ice

5.3 Setup and Reagent Preparation

1. Place a 24-well tissue culture-treated plate in a 37°C incubator for at least 1 hour.
2. Thaw ~40 µL of Corning® Matrigel® on ice for each dome to be seeded. Keep Matrigel® on ice when handling to prevent it from solidifying.
3. Prepare complete HepatiCult™ OGM (section 3.2).

   Note: ~2.25 mL of complete HepatiCult™ OGM is required for organoid expansion for 1 week in each dome. The volume of complete OGM supplied in one kit is sufficient to expand organoids in ~44 wells of a 24-well plate.
4. Prepare 50 mL of AdvDMEM + BSA as follows:
   a. Combine 48 mL Advanced DMEM/F-12 and 2 mL 25% BSA in water. Mix thoroughly.
   b. Store on ice and use cold.

   Note: This is sufficient volume to passage one full 24-well plate. If not used immediately, store at 2 - 8°C for up to 1 month.

5.4 Passaging Protocol

Complete all steps in section 5.3 before beginning the passaging protocol.

1. Check that the Matrigel® domes to be passaged are intact (i.e. the whole dome remains attached to the plate and no loose Matrigel® pieces or organoids are seen in the well). If the dome is intact, proceed to step 2. If the dome is loose, add cold AdvDMEM + BSA to top up the total volume in the well to 1 mL and let sit for 1 minute; proceed to step 4.
2. Without touching the dome, aspirate and discard the medium in each well to be passaged.
3. Using a 1 mL pipettor, forcefully add 1 mL of cold AdvDMEM + BSA to the center of each dome and let sit for 1 minute.
4. Using a 1 mL pipette tip on the pipettor, **vigorously** pipette the total volume in the well up and down 45 times, taking care not to generate bubbles.

   Note: This results in mechanical dissociation of organoids and Matrigel® into smaller fragments of 30 - 100 µm. Check fragment sizes using a light microscope; if most fragments are larger than 100 µm, triturate until they are ≤ 100 µm.

   *Note: An Integra 6-channel VOYAGER II electronic pipette (50 - 1250 µL) can also be used for this step. First, attach the 1250 µL pipette tip to the Integra pipette. Then attach a 200 µL pipette tip to the 1250 µL pipette tip. Use the 'Pipette/Mix' function for **60** - **75** cycles with the following settings: Aspirate speed = 10; Mix speed = 10; Aspirate and Mix volume = 950 µL.*
5. For every well that is being passaged, thoroughly mix fragments in suspension either by gently vortexing the plate at medium speed or using a pipette tip to stir the contents of the well **without** resuspending or pipetting to mix. Immediately transfer 3 x 10 µL of fragment suspension into an empty well of a 6-well plate to create three separate droplets. Place the plate containing the remaining fragments on ice.
6. Determine the number of organoid fragments using the droplets in the 6-well plate, as follows:
   a. Using a light microscope, count the number of organoid fragments in each 10 µL droplet. Only count fragments that are 30 - 100 µm.

      Note: If the fragment density is too high to count, dilute the suspension using AdvDMEM + BSA and repeat the count.
   b. Calculate the volume required to transfer 1000 fragments/well to the next passage.

      Note: Optimization of seeding densities per donor line is recommended. The indicated density supports a 7- to 10-day passaging schedule, on average, for most donor lines.

      Example:
      3 x 10 µL fragment counts = 35, 40, 42 fragments
      Average fragment count per 10 µL = 39 fragments
      Volume required to transfer 1000 fragments to next passage = 256 µL
   c. For each new Matrigel® dome to be seeded, add this calculated volume to a 15 mL conical tube containing 1 mL of cold AdvDMEM + BSA.
7. Centrifuge tubes containing fragments at 290 x *g* for 5 minutes. Aspirate as much of the supernatant as possible without disturbing the pellets, leaving 5 - 10 µL in the tube (the pellet is often not visible). Place tubes on ice.
8. Remove the 24-well plate from the incubator and place in a biosafety cabinet.
9. Process one tube/pellet at a time, as described below. Work quickly to ensure the Matrigel® does not solidify. Pipette tips can be cooled when working with Matrigel® to help minimize premature solidifying.

   Note: The 8 wells in the center of a 24-well plate are the most suitable for domes since their surfaces are the most even. Wells at the edges of the plate are often slightly slanted, contributing to domes touching the wall of the well and flattening out.

a. Using a pipettor with a 200 µL pipette tip, add 30 µL of thawed Matrigel® on top of the pellet. Without generating bubbles, gently mix the duct-Matrigel® suspension by pipetting up and down 5 - 8 times, going to only the first stop of the pipettor.
b. Set the pipettor volume to 40 µL. Add the entire suspension to the center of one well of the 24-well plate to form a dome. While dispensing, gradually move the pipette tip upward so that the ducts are evenly distributed throughout the dome. Dispense only to the first stop of the pipettor to avoid generating bubbles on top of the dome.

10. Repeat step 9 for the remaining pellets.
11. Place the lid on the culture plate. Carefully place the plate in an incubator at 37°C and 5% CO_2 for 10 minutes to let domes solidify.
12. Remove the plate from the incubator and place in the biosafety cabinet.
13. Without disturbing the domes, carefully add 750 µL of room temperature complete HepatiCult™ OGM against the side of each well containing a dome. Do not pipette directly onto the domes.
14. Add sterile D-PBS to any unused wells. Place the lid on the culture plate.
15. Incubate the plate at 37°C and 5% CO_2.
16. Perform a full-medium change every 2 - 3 days by carefully aspirating the medium and adding 750 µL of fresh complete HepatiCult™ OGM at room temperature.

    Note: If Matrigel® domes are loose, remove 500 µL of medium from the well, then add 500 µL of fresh medium.

    Note: To avoid weekend medium changes, perform medium changes on Mondays, Wednesdays, and Fridays.
17. Organoids will be ready to passage after ~1 week. Refer to Figure 2 for representative images.

    Note: Monitor hepatic progenitor organoids daily; they should be passaged before the lumen turns dark and organoids collapse. Cultures require passaging every 7 - 10 days.
18. Repeat steps 1 - 16 for each passage. If differentiation to mature hepatic organoids is desired, perform at least one passage as described in steps 1 - 16, then proceed to section 5.4.1.

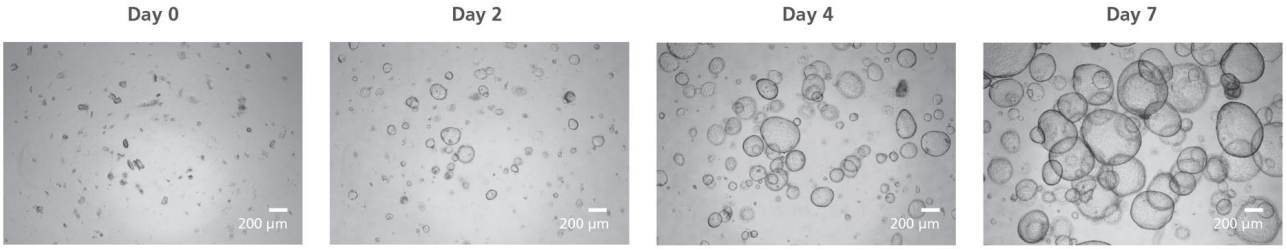


Figure 2. Expansion of Human Hepatic Organoids in Complete HepatiCult™ OGM. Organoids are ready to passage when they resemble organoids shown on Day 7. Magnification: 2X.

5.4.1 Passaging for Differentiation

1. If **differentiation** to mature hepatic organoids is desired, perform at least one passage after initiating organoids as described in section 5.4 steps 1 - 16, then perform a passage as described below. Refer to section 6.3 for an overview of the differentiation protocol.

   Note: It is recommended to optimize organoid differentiation protocol parameters for every donor and assay of interest. Refer to section 6.4.1 for differentiation protocol optimization guidelines.

   a. Perform section 5.4 steps 1 - 5.

b. In step 6, count fragments as described. Calculate the volume required to transfer 2000 fragments/well. For each new Matrigel® dome to be seeded, add this calculated volume to a 15 mL conical tube containing 1 mL of cold AdvDMEM + BSA.

c. Proceed with steps 7 - 15, seeding 2000 fragments per dome in complete HepatiCult™ OGM.

Note: It is recommended to seed additional domes in a separate plate in complete HepatiCult™ OGM for use as progenitor organoid controls/references in downstream analyses, processing wells when cultures are confluent (7 - 10 days after seeding).

Note: At least 4 confluent domes are required for RNA extraction from progenitor organoids in complete HepatiCult™ OGM (controls/references). At least 6 confluent domes are required for RNA extraction from organoids differentiated using complete HepatiCult™ ODM. The organoids from these domes are pooled prior to lysis and subsequent RNA isolation.

d. Perform a full-medium change on all wells on day 3 by carefully aspirating the medium and adding 750 µL of fresh complete HepatiCult™ OGM at room temperature.

2. On day 5 post seeding, proceed to section 6.0 for differentiation.

6.0 Differentiation of Hepatic Organoids

The following protocol is for differentiation of human hepatic organoids that have been passaged as described in section 5.4.1.

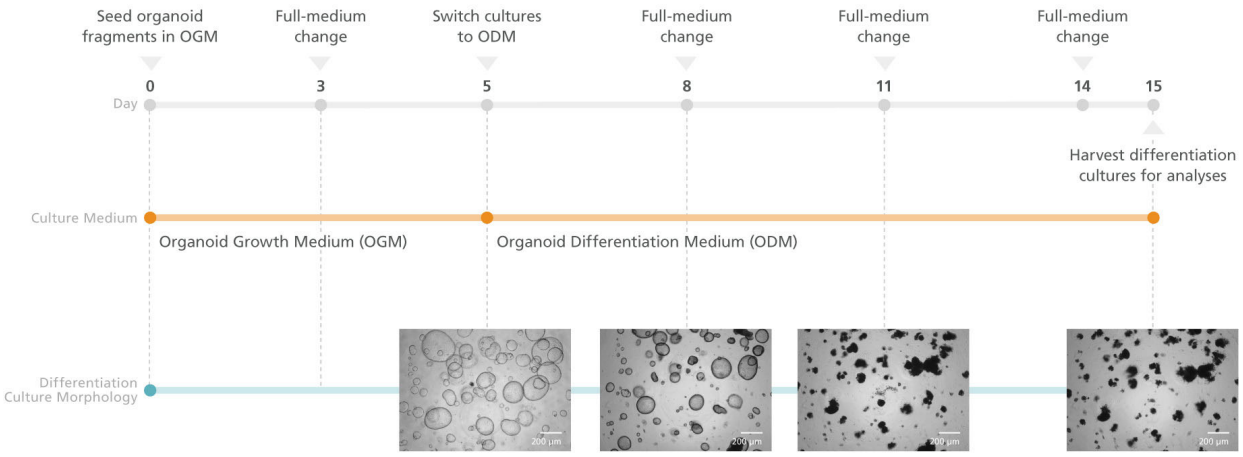
6.1 Materials Required

PRODUCT	CATALOG #
HepatiCult™ Organoid Growth Medium (Human)	100-0385
HepatiCult™ Organoid Differentiation Medium (Human)	100-0383
Dexamethasone	72092
Dimethylsulfoxide (DMSO)	e.g. Sigma D2438
Antibiotics (e.g. gentamicin)	---
24-well tissue culture-treated plate	e.g. 38017
DMEM/F-12 + 15 mM HEPES	36254

6.2 Equipment Required

- Biohazard safety cabinet certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- Pipettors (e.g. Catalog #38058) with appropriate tips
- Inverted microscope
- Refrigerator (2 - 8°C)

6.3 Protocol Diagram



Perform at least one passage as described in section 5.4 steps 1 - 16, then seed organoid fragments in complete HepatiCult™ OGM (section 5.4.1). On day 3 post seeding, perform a full-medium change with complete HepatiCult™ OGM. On day 5, switch to complete HepatiCult™ ODM (section 6.4). Perform a full-medium change with complete HepatiCult™ ODM on days 8, 11, and 14. On day 15, harvest/process wells for characterization and functional assays.

6.4 Differentiation Protocol

For wells that will be differentiated, proceed to step 1 on day 5 post seeding:

1. Prepare complete HepatiCult™ ODM (section 3.3).

   Note: ~3 mL of complete HepatiCult™ ODM is required for each dome being differentiated. The volume of complete ODM supplied in one kit is sufficient to differentiate organoids in ~33 wells of a 24-well plate.

2. Aliquot the required volume of complete HepatiCult™ ODM and warm to room temperature (15 - 25°C).

   Note: Complete HepatiCult™ ODM is light sensitive; minimize exposure to light.

3. Change medium to complete HepatiCult™ ODM on day 5 post seeding as follows:
   a. Without touching the dome, carefully aspirate complete HepatiCult™ OGM from wells and discard.
   b. Wash domes by adding 750 µL of room temperature DMEM/F-12 + 15 mM HEPES to all domes. Carefully aspirate and discard this volume without touching the dome.
   c. Add 750 µL of room temperature complete HepatiCult™ ODM to each well.
   d. Incubate at 37°C.
4. Perform a full-medium change every 3 days using complete HepatiCult™ ODM at room temperature, and excluding the wash with DMEM/F-12 + 15 mM HEPES (total of 3 full-medium changes on days 8, 11, and 14). For assays investigating secretion functions using the spent medium, it is recommended to perform the final full-medium change on day 13 (i.e. 48 hours before harvest) instead of day 14.

   Note: To monitor organoid differentiation, take photos of the same field of view every 2 - 3 days until the end of the differentiation protocol.

   Note: If Matrigel® domes are loose, remove 500 µL of medium from the well, then add 500 µL of fresh medium.

   Note: Some cellular debris may be observed in the spent medium. This can be aspirated and discarded.

5. On day 15, harvest/process wells for characterization and functional assays.

6.4.1 Differentiation Protocol Optimization Guidelines

The following protocol parameters should be optimized for every donor and assay of interest; recommended ranges for preliminary testing are also provided.

- Seeding density per dome for differentiation cultures
  - Cell turnover is observed during hepatic organoid differentiation. Optimal seeding densities will support the generation of enough viable cells at the end of the differentiation to use in downstream analyses.
  - Recommended seeding densities to test: 1000 - 2500 fragments per dome
- Number of domes to be seeded for differentiation assays
  - Intracellular protein- and nucleic acid-based analyses require more differentiated organoid material, while assays investigating enzymatic activity or secretory functions can be performed on fewer wells
  - Recommended testing ranges: 3 - 6 wells per donor per differentiation
- Duration of culture in complete HepatiCult™ OGM prior to differentiation using ODM
  - Organoids should be differentiated once they have started to expand, but before they begin to collapse or deflate
  - Recommended testing range: Culture in complete HepatiCult™ OGM for 3 - 5 days
- Duration of differentiation in complete HepatiCult™ ODM and harvest timepoint

- Some functionalities and features of mature hepatic cells may be observed as early as day 11 of the differentiation protocol, and earlier harvest timepoints may support higher viable cell yields
- Recommended harvest timepoints to test: Day 10 - 15

7.0 Appendix: Passaging Human Hepatic Organoids to 96-Well Plates

The following protocol is for passaging human hepatic organoids from dome cultures in 24-well plates (established in section 4.0 or from cryopreserved sources) to dome cultures in 96-well plates.

7.1 Materials Required

PRODUCT	CATALOG #
HepatiCult™ Organoid Growth Medium (Human)	100-0385
Antibiotics (e.g. gentamicin)	---
Anti-Adherence Rinsing Solution	07010
Advanced DMEM/F-12	Thermo Fisher 12634028
96-well tissue culture-treated plate	Corning 3596
6-well tissue culture-treated plate	38016
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free, LDEV-free (≥ 8 mg/mL protein)	Corning 356231
25% Bovine serum albumin (BSA) solution in water	---
D-PBS (Without Ca++ and Mg++)	37350
2 mL, 5 mL, and 10 mL serological pipettes	e.g. 38004 and 38005
15 mL and 50 mL conical tubes	e.g. 38009 and 38010
Reversible Strainers, Large, 37 µm and 70 µm	27250 and 27260

7.2 Equipment Required

- Biohazard safety cabinet certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- Pipettors (e.g. Catalog #38058) with appropriate tips
- Inverted microscope
- Refrigerator (2 - 8°C)
- Styrofoam box with ice

7.3 Setup and Reagent Preparation

1. Place a 96-well tissue culture-treated plate in a 37°C incubator for at least 1 hour (overnight warming is recommended).
2. Thaw ~700 µL of Corning® Matrigel® on ice to seed one full 96-well plate. Keep Matrigel® on ice when handling to prevent it from solidifying.
3. Prepare complete HepatiCult™ OGM (section 3.2).

   Note: ~0.6 mL of complete HepatiCult™ OGM is required for organoid expansion for 1 week in each dome. The volume of complete OGM supplied in one kit is sufficient to expand organoids in ~166 wells of a 96-well plate.

4. Prepare 50 mL of AdvDMEM + BSA as follows:
   a. Combine 48 mL Advanced DMEM/F-12 and 2 mL 25% BSA in water. Mix thoroughly.
   b. Store on ice and use cold.

   Note: This is sufficient volume to passage one full 96-well plate. If not used immediately, store at 2 - 8°C for up to 1 month.
5. Optional: Hepatic organoid fragments may adhere to the surfaces of conical tubes and serological pipettes. To minimize this adherence, pre-wet 4 x 50 mL and 2 x 15 mL conical tubes with Anti-Adherence Rinsing Solution and Advanced DMEM/F-12, as follows:
   a. Transfer 5 mL of Anti-Adherence Rinsing Solution to a 15 mL conical tube (or 10 mL of Rinsing Solution to a 50 mL conical tube).
   b. Tighten the lids and swirl tubes to coat. Transfer the Rinsing Solution to the remaining tubes, swirling to coat each one.
   c. Aspirate Rinsing Solution from tubes. Repeat steps a and b with Advanced DMEM/F-12, then aspirate residual Advanced DMEM/F-12.
   d. Cap all coated tubes and store on ice until required.

7.4 Passaging Protocol

Complete all steps in section 7.3 before beginning the passaging protocol.

1. Check that the Matrigel® domes to be passaged are intact (i.e. the whole dome remains attached to the plate and no loose Matrigel® pieces or organoids are seen in the well). If the dome is intact, proceed to step 2. If the dome is loose, add cold AdvDMEM + BSA to top up the total volume in the well to 1 mL and let sit for 1 minute; proceed to step 4.
2. Without touching the dome, aspirate and discard the medium in each well to be passaged.
3. Using a 1 mL pipettor, forcefully add 1 mL of cold AdvDMEM + BSA to the center of each dome and let sit for 1 minute.
4. Using a 1 mL pipette tip on the pipettor, **vigorously** pipette the total volume in the well up and down 45 times, taking care not to generate bubbles.

   Note: This results in mechanical dissociation of organoids and Matrigel® into smaller fragments of 30 - 100 µm. Check fragment sizes using a light microscope; if most fragments are larger than 100 µm, triturate until they are ≤ 100 µm.

   *Note: An Integra 6-channel VOYAGER II electronic pipette (50 - 1250 µL) can also be used for this step. First, attach the 1250 µL pipette tip to the Integra pipette. Then attach a 200 µL pipette tip to the 1250 µL pipette tip. Use the 'Pipette/Mix' function for **60** - **75** cycles with the following settings: Aspirate speed = 10; Mix speed = 10; Aspirate and Mix volume = 950 µL.*
5. Transfer the entire volume of fragment suspension from each well to a pre-wetted 50 mL conical tube on ice.
6. Wash the wells from which the organoids were harvested with 1 mL cold AdvDMEM + BSA. Add this wash volume to the fragment suspension.
7. Place a 70 µm reversible strainer on a new pre-wetted 50 mL conical tube. Pre-wet the surface of the strainer with 2 mL Rinsing Solution followed by 2 mL AdvDMEM + BSA.
8. Using a 10 mL serological pipette, transfer 10 mL of the fragment suspension through the 70 µm strainer. Wash off fragments > 70 µm (collected on the strainer) as described below before passing an additional 10 mL of fragment suspension (from step 6) through the strainer. Continue until the entire volume of fragment suspension has been passed through the strainer. Place the flow-through on ice. Proceed to step 9.

To wash off fragments > 70 µm:

a. Reverse the 70 µm strainer(s) onto a new pre-wetted 50 mL conical tube. Using a 5 mL serological pipette, add 2 - 5 mL of AdvDMEM + BSA to the reversed strainer, ensuring the tip of the serological pipette touches the surface of the membrane to wash off all fragments. Replace the strainer on the 50 mL conical tube and continue with step 8.

b. *Optional: To maximize the yield of fragments available to seed domes in the 96-well plate, fragments that are > 70 µm in size can be triturated further and collected as follows:*

   i. Aliquot 1 mL of the suspension of fragments > 70 µm into each well of a new 24-well plate and further triturate as described in step 4.

   ii. Pass the triturated fragment suspension through another pre-wetted 70 µm strainer and place the flow-through on ice. Pool this flow-through with the flow-through collected in step 8.

9. Place a 37 µm reversible strainer on a new 50 mL conical tube (this tube does not need to be pre-wetted). Pre-wet the surface of the strainer with 2 mL Rinsing Solution followed by 2 mL AdvDMEM + BSA.
10. Using a 10 mL serological pipette, transfer 5 mL of flow-through from step 8 through the 37 µm strainer. Discard the flow-through.

    Note: The fragment suspension passes through the 37 µm very slowly due to the high density of fragments in the suspension. It is important to wash fragments off the strainer as described in step 11 after 5 mL of suspension has been filtered.
11. Reverse the 37 µm strainer onto a new pre-wetted 50 mL conical tube. Using a 5 mL serological pipette, add 2 - 5 mL of AdvDMEM + BSA to the reversed strainer, ensuring the tip of the serological pipette touches the surface of the membrane to wash off and collect all fragments. Discard the strainer.
12. Repeat steps 9 - 11 until all of the flow-through from step 8 has been passed through the 37 µm strainer, and all the collected fragments > 37 µm in size have been washed off, pooling these fragments into the same 50 mL conical tube used in step 11.
13. Transfer 3 x 10 µL of fragment suspension into an empty well of a 6-well plate to create three separate droplets. Place the tube containing the remaining fragments on ice.
14. Determine the number of organoid fragments using the droplets in the 6-well plate, as follows:

    a. Using a light microscope, count the number of organoid fragments in each 10 µL droplet. Count all fragments.

       Note: If the fragment density is too high to count, dilute the suspension using AdvDMEM + BSA and repeat the count.

    b. Determine the volume required to seed the desired number of wells in the 96-well plate.

       Note: Seeding density should be optimized per donor. Seeding with 175 - 350 fragments per dome per well of the 96-well plate is recommended.

       Example:
       3 x 10 µL fragment counts = 35, 40, 42 fragments
       Average fragment count per 10 µL = 39 fragments
       Volume to seed 250 fragments/well in 1 dome in a 96-well plate = 64 µL
       Volume to seed 250 fragments/well in 96 domes in a 96-well plate = 6.154 mL
       Volume to transfer to 15 mL conical tube, including 15% extra volume = 7.077 mL
15. Transfer the calculated volume to a pre-wetted 15 mL conical tube.
16. Centrifuge the tube containing fragments at 290 x *g* for 5 minutes. Aspirate as much of the supernatant as possible without disturbing the pellet.

17. Resuspend fragments in 6 µL of Matrigel® per dome to be seeded, plus an extra 15% to account for pipetting loss. Using a 1 mL pipettor, mix gently, taking care not to introduce bubbles. Place tube on ice.

    Example:

    Volume of Matrigel® to seed 96 domes = 576 µL
    Volume of Matrigel® to add to pellet, including 15% extra volume = 662 µL

18. Remove the 96-well plate from the incubator and place in a biosafety cabinet.
19. Pipette 6 µL of the fragment-Matrigel® suspension into the centre of each well to form a dome. After plating every 6 - 12 domes, return the fragment-Matrigel® suspension to ice for 30 seconds. Mix gently with a 1 mL pipettor before proceeding with plating an additional 6 - 12 domes.

    Note: We recommend using a single-channel electronic repeater pipette and seeding domes across rows of the 96-well plate. Some domes may slide to the edges of the well and flatten slightly; robust hepatic organoid expansion is still observed in these wells, and they can be used for downstream analyses without issue.

20. Place the lid on the culture plate. Carefully place the plate in an incubator at 37°C and 5% CO_2 for 10 minutes to let domes solidify.
21. Remove the plate from the incubator and place in the biosafety cabinet.
22. Without disturbing the domes, carefully add 200 µL of room temperature complete HepatiCult™ OGM against the side of each well containing a dome. Do not pipette directly onto the domes.
23. Add sterile D-PBS to any unused wells. Place the lid on the culture plate.
24. Incubate the plate at 37°C and 5% CO_2 until ready to use. Monitor medium levels, performing a full-medium change every 2 - 3 days by carefully removing 150 µL of medium from wells and adding 200 µL of fresh complete HepatiCult™ OGM at room temperature.
25. Organoids can be further differentiated in HepatiCult™ Organoid Differentiation Medium and used for downstream analyses as described in section 6.0, using 200 µL for washing and per full-medium change with fresh HepatiCult™ Organoid Differentiation Medium (section 6.4 step 3).

TECHNICAL MANUAL

Initiation, Growth, and Differentiation of Human Hepatic Organoids Using HepatiCult™

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