TECHNICAL MANUAL

Establishment, Growth, and Differentiation of Human Pluripotent Stem Cell-Derived Hepatic Organoids Using STEMdiff™ Hepatic Organoid Media

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1.0 Introduction

Hepatic organoids are three-dimensional (3D) cell culture systems that model features of in vivo hepatocytes, recapitulate donor heterogeneity, and therefore serve as a valuable model for studying liver cell biology. They are a proliferative and physiologically relevant alternative to conventional two-dimensional (2D) cell culture for screening applications and studying hepatic development, regeneration, detoxification, metabolism, and disease. STEMdiff™ Hepatic Organoid Growth and Differentiation Media support the serum-free culture of hepatic organoids from human pluripotent stem cell (hPSC)-derived hepatic progenitors (HPs) and/or hepatocyte-like cells (HLCs), generated using STEMdiff™ Hepatocyte Kit (Catalog #100-0520).

This organoid culture workflow includes robust organoid establishment, long-term expansion, and scale-up of established organoids in complete STEMdiff™ Hepatic Organoid Growth Medium (OGM; Catalog #100-1773), and differentiation of expanded organoids in complete STEMdiff™ Hepatic Organoid Differentiation Medium (ODM; Catalog #100-1774) to generate organoids that exhibit mature hepatic phenotypes. Organoids cultured in complete STEMdiff™ Hepatic OGM and ODM can be adapted to a range of culture formats including dilute Matrigel® suspension cultures, organoid-derived monolayers, and 96- and 384-well culture plates for higher throughput assays. They also remain viable following cryopreservation in CryoStor® CS10, retaining their capacity for proliferation in STEMdiff™ Hepatic OGM and further differentiation using STEMdiff™ Hepatic ODM.

2.0 Materials, Reagents, and Equipment

2.1 STEMdiff™ Hepatic Organoid Growth and Differentiation Media

The components listed below are available as part of STEMdiff™ Hepatic Organoid Growth Medium or STEMdiff™ Hepatic Organoid Differentiation Medium and are not available for individual sale.

For component storage and stability information, refer to the product information sheet for STEMdiff™ Hepatic OGM and ODM (Document #10000031145), available at www.stemcell.com, or contact us to request a copy.

PRODUCT NAME	CATALOG #	COMPONENT NAME	COMPONENT #	QUANTITY
STEMdiff™ Hepatic Organoid Growth Medium	100-1773	STEMdiff™ Hepatic Organoid Basal Medium	100-1770	95 mL
		STEMdiff™ Hepatic Organoid Growth Supplement*	100-1771	5 mL
STEMdiff™ Hepatic Organoid Differentiation Medium	100-1774	STEMdiff™ Hepatic Organoid Basal Medium	100-1770	95 mL
		STEMdiff™ Hepatic Organoid Differentiation Supplement*	100-1772	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-I antibodies and/or HIV-I antigen. However, this product should be considered potentially infectious and treated in accordance with universal handling precautions.

2.2 Additional Required Materials and Reagents

PRODUCT	CATALOG #
6-well tissue culture-treated plate	e.g. 38016
24-well tissue culture-treated plate OR 24-Well Organoid Culture Plates	e.g. 38017 OR 200-0561
15 mL and 50 mL conical tubes	e.g. 38009 and 38010
Advanced DMEM/F-12	Thermo Fisher 12634028
Antibiotics (e.g. gentamicin)	---
Bovine serum albumin (BSA)	---
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free, LDEV-free (≥ 8 mg/mL protein)	Corning 356231
Dexamethasone	72092
Dimethyl sulfoxide (DMSO)	e.g. Sigma D2438
DMEM/F-12 + 15 mM HEPES	36254
D-PBS (Without Ca++ and Mg++)	37350
STEMdiff™ Hepatocyte Kit	100-0520
OPTIONAL: Anti-Adherence Rinsing Solution	07010

2.3 Equipment Required

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

3.0 Establishment of Hepatic Organoids

The following protocol is for establishing hepatic organoids from hPSC-derived hepatic progenitors (HPs) and/or hepatocyte-like cells (HLCs; recommended), generated using the STEMdiff™ Hepatocyte Kit, in Matrigel® domes/layers. For generating HPs and/or HLCs, refer to the product information sheet for STEMdiff™ Hepatocyte Kit (Document #10000011543). After generating HPs and/or HLCs (10- or 21-day protocol), proceed with hepatic organoid establishment.

3.1 Protocol Diagram

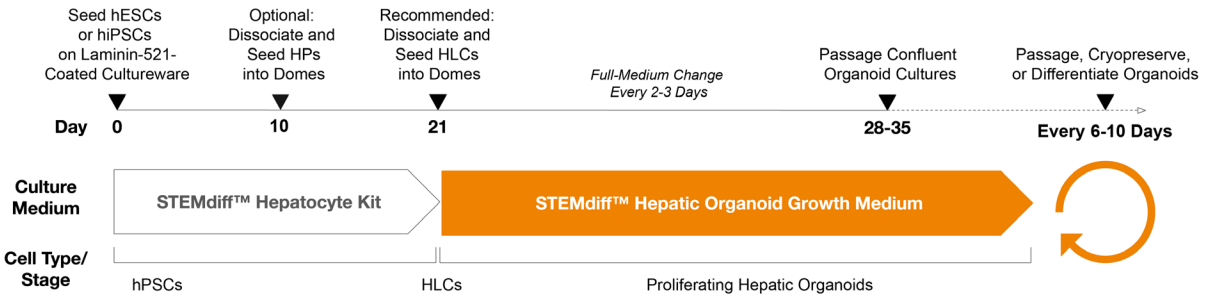

Figure 1. Protocol Diagram for Establishment of Hepatic Organoids. Day-10 HPs or day-21 HLCs, generated from human embryonic or induced pluripotent stem cells (hESCs or hiPSCs) using STEMdiff™ Hepatocyte Kit, are dissociated, seeded in Matrigel® domes/layers, and cultured in STEMdiff™ Hepatic OGM to generate confluent organoid cultures within 7 to 14 days. On average, organoids are ready for passaging on day 10, but cultures should be passaged when confluent, any time between day 7 - 14.

3.2 Setup and Reagent Preparation

Complete the setup and reagent preparation below when HPs and/or HLCs are ready to be used for establishing hepatic organoids.

3.2.1 Preparing Complete STEMdiff™ Hepatic Organoid Growth Medium (OGM)

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

Note: 0.5 mL of complete STEMdiff™ Hepatic OGM is required per medium change for each well to be seeded.

1. Thaw STEMdiff™ Hepatic Organoid Growth Supplement overnight at 2 - 8°C. Mix well.

   Note: If not using immediately, aliquot and store at -20°C. Do not exceed the shelf life of the supplement. Do not re-freeze aliquots after thawing.
2. Add 5 mL of Growth Supplement to 95 mL of Basal Medium.
3. Add antibiotics (e.g. final concentration 50 µg/mL gentamicin).
4. Mix well. Warm to room temperature (15 - 25°C) before use.

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

3.2.2 Preparing Cultureware and Matrigel®

1. Place a 24-well tissue culture-treated plate in a 37°C incubator for at least 1 hour.

   Note: If using a 24-well *Organoid Culture Plate, use plates at room temperature (15 - 25°C); pre-warming is not required.*

2. Thaw ~40 µL of Matrigel® on ice for each dome to be seeded. If using an Organoid Culture Plate, thaw 60 µL of Matrigel® for each well to be seeded. One well of HPs or HLCs generated in a 24-well plate is used to seed 4 wells of a 24-well plate or Organoid Culture Plate.

   Note: Keep Matrigel® on ice when handling to prevent it from solidifying.

3.3 Organoid Establishment Protocol

The following instructions are for organoid establishment from HPs and/or HLCs (recommended) generated in 24-well plates. If using other cultureware, adjust volumes accordingly.

1. Without aspirating the medium, use a pipettor with a 200 µL or 1000 µL pipette tip to manually scrape HPs or HLCs off the bottom of each well.
2. Transfer the entire contents of each well into a 15 mL conical tube.

   Note: The contents of up to two wells can be pooled in one tube.

3. Pipette the total suspension volume up and down 2 - 3 times to generate fragments that are 30 - 100 µm in diameter.
4. Centrifuge the tube at 300 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.
5. Remove the warmed 24-well plate from the incubator and place in a biosafety cabinet.
   If using an Organoid Culture Plate, proceed to step 6.
6. 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 tissue-culture treated plate are the most suitable for plating 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 120 µL of thawed Matrigel® for each well of HPs or HLCs to be seeded (i.e. 30 µL for each dome to be seeded) on top of the pellet. Without generating bubbles, gently mix the fragment-Matrigel® suspension by pipetting up and down 5 - 8 times, dispensing only to the first stop of the pipettor.

      If using an Organoid Culture Plate, add 200 µL of thawed Matrigel® for each well of HPs or HLCs to be seeded (i.e. 50 µL for each layer to be seeded).

   b. Set the pipettor volume to 30 µL. To form domes, transfer 30 µL of fragment-Matrigel® suspension to the center of each of 4 or 8 wells of the 24-well plate. While dispensing, gradually move the pipette tip upward so that the fragments are evenly distributed throughout the dome. Dispense only to the first stop of the pipettor to avoid generating bubbles.

      If using an Organoid Culture Plate, set the pipettor volume 50 µL. To seed matrix layers, transfer 50 µL of fragment-Matrigel® suspension to the center of the well, using the pipette tip to drag the suspension around the edges if required to fully fill any gaps. Dispense only to the first stop of the pipettor to avoid generating bubbles.

7. Repeat step 6 for the remaining pellets/tubes.
8. 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 Matrigel® solidify.
9. Remove the plate from the incubator and place in the biosafety cabinet.

10. Without disturbing the matrix, carefully add 500 µL of room temperature (15 - 25°C) complete STEMdiff™ Hepatic OGM against the side of each well containing a dome/layer. Do not pipette directly onto the domes/layer.
11. Add sterile D-PBS (Without Ca++ and Mg++) to any unused wells. Place the lid on the culture plate. Incubate at 37° C and 5% CO_2.
12. Perform a full-medium change every 2 - 3 days by carefully aspirating the medium and adding 500 µL of fresh room temperature complete STEMdiff™ Hepatic OGM to each well containing a dome/layer.

    Note: If Matrigel® domes/layers are loose, remove 400 µL of medium from the well, then add 400 µL of fresh medium. To avoid weekend medium changes, perform medium changes on Mondays, Wednesdays, and Fridays.

    Note: To monitor organoid growth, take images of the same field of view every 2 - 3 days until they are passaged.
13. Passage organoids before the lumen turns dark and the organoids collapse (up to 2 weeks), as described in section 4.2 below. Refer to Figure 2 for representative images.

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

    - *Prepare reagents (section 4.1) and generate organoid fragments (section 4.2 steps 1 - 4).*
    - *Transfer the entire volume of fragment suspension to a 15 mL conical tube containing 1 mL of cold Advanced DMEM + BSA.*
    - *Proceed to section 4.2 step 7.*

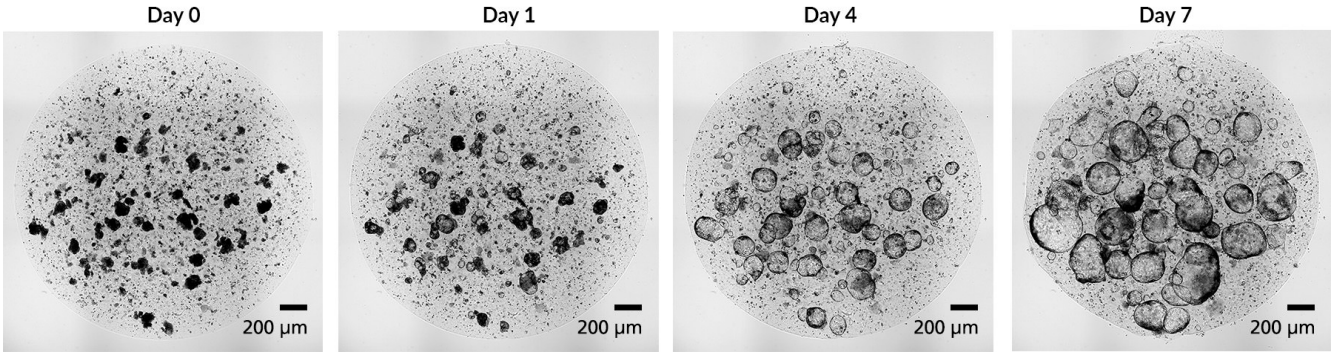

Figure 2. Establishment of hPSC-Derived Hepatic Organoids. Organoids established from HLCs in complete STEMdiff™ Hepatic OGM are ready to passage when they resemble the organoids shown on day 7.

4.0 Growth of Hepatic Organoids

The following protocol is for passaging human hepatic organoids in dome/layer cultures (established in section 3.0 or from cryopreserved sources) in 24-well tissue culture-plates or 24-well Organoid Culture Plates.

4.1 Setup and Reagent Preparation

Place a new 24-well tissue culture-treated plate in a 37°C incubator for at least 1 hour.
Note: If using a 24-well *Organoid Culture Plate, use plates at room temperature (15 - 25°C); pre-warming is not required.*

14. Thaw ~40 µL of Matrigel® on ice for each dome to be seeded. If using an Organoid Culture Plate, thaw 60 µL of Matrigel® for each well to be seeded.
    Note: Keep Matrigel® on ice when handling to prevent it from solidifying.
15. Prepare complete STEMdiff™ Hepatic OGM (section 3.2.1).
16. Prepare 50 mL of Advanced DMEM/F-12 + BSA as follows:
    a. Combine 48 mL of Advanced DMEM/F-12 and 2 mL of 25% BSA in water. Mix thoroughly.
    b. Store on ice and use cold.

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

4.2 Passaging Protocol

Complete all steps in section 4.1 before beginning the passaging protocol.

1. Check that the Matrigel® domes/layers to be passaged are intact (i.e. no loose Matrigel® pieces or organoids are seen in the well). If the dome/layer is intact, proceed to step 2. If the dome/layer is loose, add cold Advanced DMEM/F-12 + 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/layer, aspirate and discard the medium in each well to be passaged.
3. Using a 1 mL pipettor, forcefully add 1 mL of cold Advanced DMEM/F-12 + BSA to the center of each dome/layer 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 at least 10 times to generate fragments of organoids and Matrigel® that are 30 - 100 µm in diameter. Take care to minimize the generation of bubbles.

   Note: The number of trituration cycles required to generate fragments of this size will vary across cell lines and passages. Check fragment sizes using a light microscope; if most fragments are larger than 100 µm, perform additional trituration cycles as required.

   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 at least 15 cycles with the following settings:

   Aspirate speed = 10; Mix speed = 10; Aspirate and Mix volume = 875 µL. Perform additional trituration cycles as required to generate fragments that are 30 - 100 µm in diameter.
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, to be used for organoid fragment counting. 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 in diameter.

Note: If the fragment density is too high to count, dilute the suspension using Advanced DMEM/F-12 + 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 cell line is recommended. The indicated density supports a 6- to 10-day passaging schedule for most cell 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/layer to be seeded, transfer a volume that contains 1000 fragments from the suspension (kept on ice in step 5) to a 15 mL conical tube containing 1 mL of cold Advanced DMEM/F-12 + BSA.

7. Centrifuge tubes containing fragments at 300 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 new 24-well tissue culture-treated plate from the incubator and place in a biosafety cabinet. If using Organoid Culture Plates, proceed to step 9.
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 tissue-culture treated *plate are the most suitable for plating 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. If using an Organoid Culture Plate, add 50 µL of thawed Matrigel®.
b. Without generating bubbles, gently mix the fragment-Matrigel® suspension by pipetting up and down 5 - 8 times, dispensing only to the first stop of the pipettor.
c. Set the pipettor volume to 40 µL. If using an Organoid Culture Plate, set the pipettor volume 60 µL.
d. Add the entire suspension to the center of one well of the 24-well plate to form a dome/layer. While dispensing, gradually move the pipette tip upward so that the fragments are evenly distributed throughout the dome/layer. Dispense only to the first stop of the pipettor to avoid generating bubbles on top of the dome/layer. If using Organoid Culture Plate, use the pipette tip to drag the suspension around the edges if required to fully fill any gaps.

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 the Matrigel® solidify.
12. Remove the plate from the incubator and place in the biosafety cabinet.

Optional: Organoids undergoing differentiation may attach to the culture plate and expand as a monolayer. When seeding organoids for differentiation, 2D attachment of cells can be minimized as follows:

a. Carefully add 250 µL of Anti-Adherence Rinsing Solution to the well(s).
b. Gently rock the plate to ensure the volume coats the entire surface of the well surrounding the dome/layer(s).
c. Carefully aspirate and discard the Anti-Adherence Rinsing Solution without disturbing the dome/layer(s).

d. Wash by adding 750 µL of room temperature sterile D-PBS (Without Ca++ and Mg++) to all wells. Carefully aspirate and discard this volume without touching the matrix.

e. Proceed to step 13.

13. Without disturbing the domes/layers, carefully add 500 µL of room temperature complete STEMdiff™ Hepatic OGM against the side of each well containing a dome/layer. Do not pipette directly onto the domes/layers.
14. Add sterile D-PBS (Without Ca++ and Mg++) 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 500 µL of fresh complete STEMdiff™ Hepatic OGM at room temperature.

    Note: If Matrigel® domes/layers are loose, remove 400 µL of medium from the well, then add 400 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 approximately 1 week. Refer to Figure 3 for representative organoid morphology images.

    Note: Monitor hepatic organoids daily; they should be passaged before the lumen turns dark and organoids collapse, usually every 6 - 10 days.
18. Repeat steps 1 - 16 for each passage.

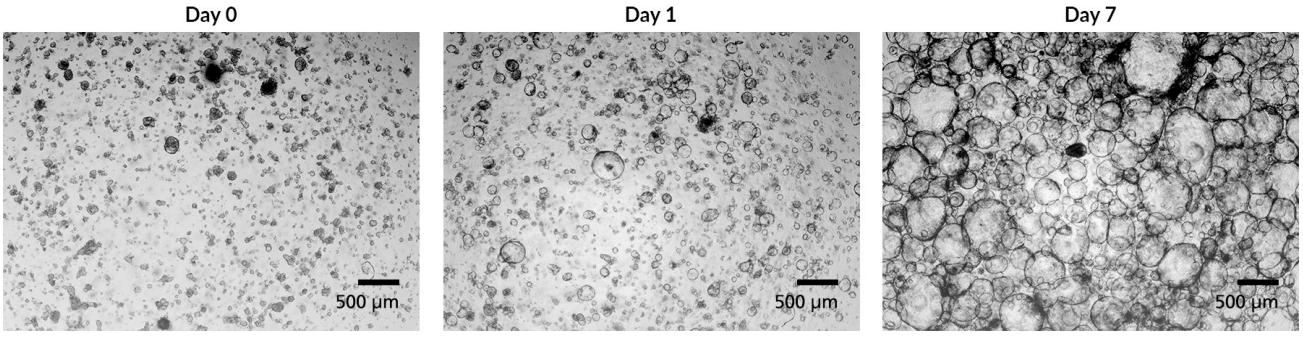

Figure 3. Expansion of hPSC-Derived Hepatic Organoids. Organoids are ready to passage when they resemble organoids shown on day 7.

5.0 Differentiation of Hepatic Organoids

The following protocol is for differentiation of human hepatic organoids that have been passaged as described in section 4.0.

5.1 Protocol Diagram

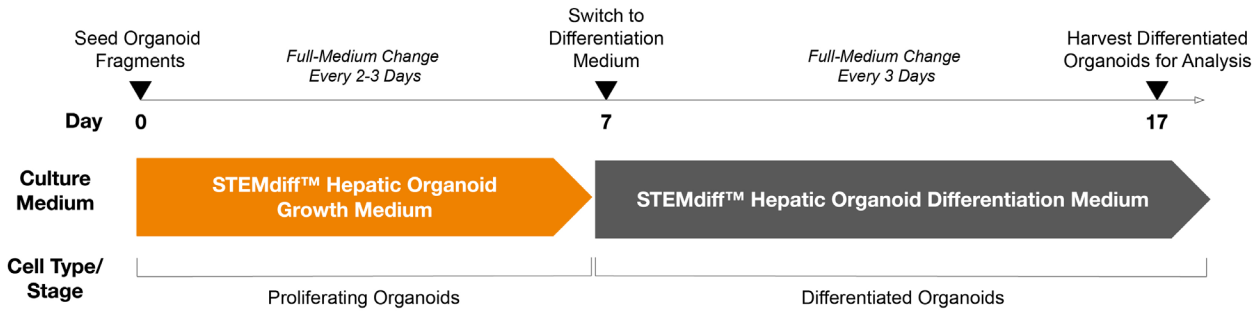

Figure 4. Protocol Diagram for Differentiation of Hepatic Organoids. Perform at least one passage as described in section 4.2 (steps 1 - 16), then seed organoid fragments in complete STEMdiff™ Hepatic OGM. On day 3 post seeding, perform a full-medium change with complete STEMdiff™ Hepatic OGM. On day 7, switch to complete STEMdiff™ Hepatic ODM (section 5.2.1). Perform a full-medium change with complete STEMdiff™ Hepatic ODM on days 10, 13, and 16. On day 17, harvest/process wells for characterization and functional assays.

5.2 Setup and Reagent Preparation

5.2.1 Preparing Complete STEMdiff™ Hepatic Organoid Differentiation Medium (ODM)

Note: Complete STEMdiff™ Hepatic ODM must be used within 2 weeks of preparation. Prepare close to the time of use (section 5.3).

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

1. Thaw STEMdiff™ Hepatic Organoid Differentiation Supplement overnight at 2 - 8°C. Mix well.

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

   Note: If not using immediately, aliquot and store at -20°C. Do not exceed the shelf life of the supplement. Do not re-freeze aliquots after thawing.
2. Add 5 mL of Differentiation Supplement to 95 mL of Basal Medium.
3. Add antibiotics (e.g. final concentration 50 µg/mL gentamicin).
4. Mix well.

   Note: If not using immediately, store complete STEMdiff™ Hepatic ODM at 2 - 8°C for up to 2 weeks. Complete STEMdiff™ Hepatic ODM is light sensitive; minimize exposure to light.

5. On days when the medium is used, aliquot the volume of complete STEMdiff™ Hepatic ODM required to perform a full-medium change in a tube and warm to room temperature. Immediately before use, add 1 µL of 3 mM dexamethasone in DMSO per mL of complete STEMdiff™ Hepatic ODM (final concentration 3 µM). Mix thoroughly.

   Note: Discard any leftover volumes of complete STEMdiff™ Hepatic ODM containing dexamethasone.

5.3 Organoid Differentiation Protocol

Prior to organoid differentiation, perform at least one passage as described in section 4.2 steps 1 - 16. Then proceed with the steps described below.

Note: It is recommended to optimize organoid differentiation protocol parameters for every hPSC line and assay of interest. Refer to section 5.3.1 for differentiation protocol optimization guidelines.

1. Seed organoid fragments for differentiation by performing an organoid passage as described in section 4.2 steps 1 - 15.

   Note: It is recommended to seed additional domes/layers in a separate plate in complete STEMdiff™ Hepatic OGM for use as undifferentiated organoid controls/references in downstream analyses. Process these wells when cultures are confluent (e.g. 6 - 10 days after seeding).

   Note: At least 2 confluent wells are required for RNA extraction from undifferentiated organoids in complete STEMdiff™ Hepatic OGM (controls/references). At least 3 confluent wells are required for RNA extraction from organoids differentiated using complete STEMdiff™ Hepatic ODM. The organoids from these wells are pooled prior to lysis and subsequent RNA isolation.
2. Perform a full-medium change on all wells on day 3 post seeding by carefully aspirating the medium and adding 500 µL of fresh complete STEMdiff™ Hepatic OGM at room temperature.
3. On day 7 post-seeding, prepare complete STEMdiff™ Hepatic ODM (section 5.2.1).

   Note: Complete STEMdiff™ Hepatic ODM is light sensitive; minimize exposure to light.
4. Change medium to complete STEMdiff™ Hepatic ODM on day 7 post seeding as follows:
   a. Without touching the dome/layer, carefully aspirate complete STEMdiff™ Hepatic OGM from wells.
   b. Wash domes/layers by adding 750 µL of room temperature DMEM/F-12 + 15 mM HEPES. Carefully aspirate and discard this volume without touching the matrix.
   c. Add 500 µL of room temperature complete STEMdiff™ Hepatic ODM to each well.
   d. Incubate the plate at 37°C and 5% CO_2.
5. Perform a full-medium change every 3 days using room temperature complete STEMdiff™ Hepatic ODM, for a total of three full-medium changes on days 10, 13, and 16. Exclude the wash with DMEM/F-12 + 15 mM HEPES.

   Note: For assays investigating secretory functions using the spent medium, it is recommended to perform the final full-medium change on day 14 or 15 (i.e. 48 - 72 hours before harvest) instead of day 16.

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

   Note: If Matrigel® domes/layers are loose, remove 400 µL of medium from the well, then add 400 µL of fresh medium.

   Note: Organoids undergo significant changes in morphology during differentiation as seen in Figure 5. Some cellular debris may be observed in the spent medium and can be aspirated and discarded.
6. On day 17, harvest/process wells for characterization and functional assays.

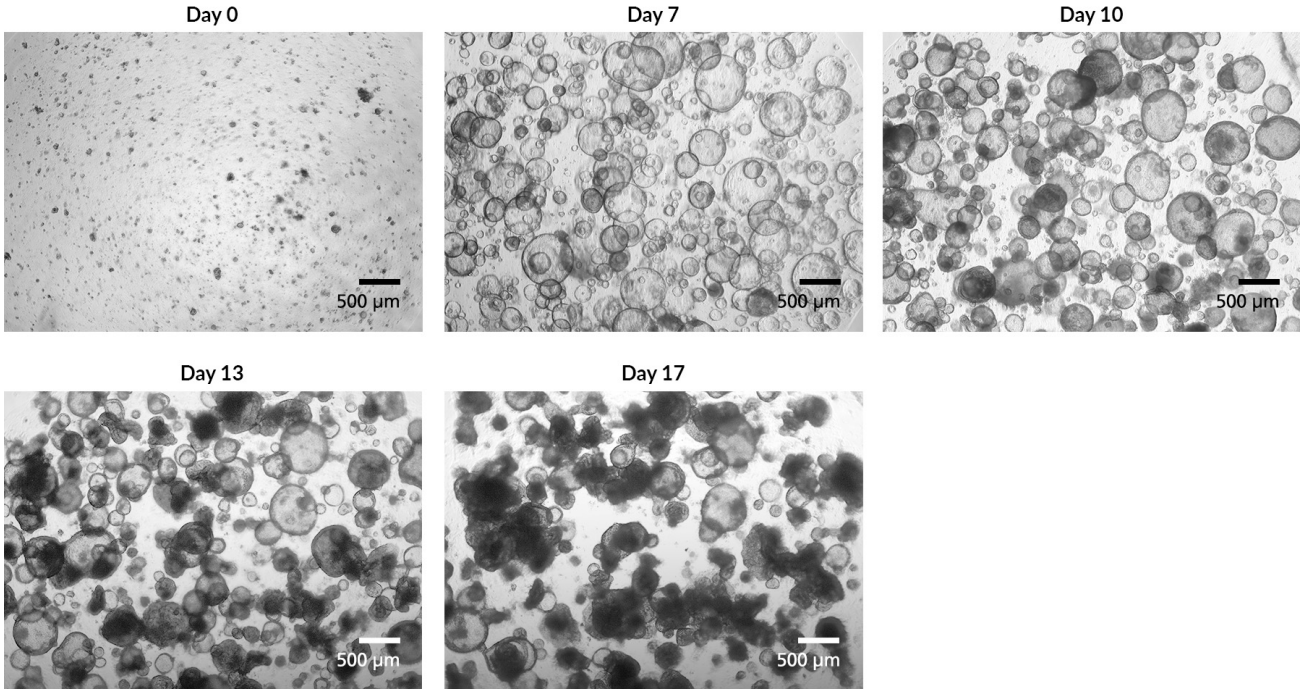

Figure 5. Differentiation of hPSC-Derived Hepatic Organoids. Organoids undergo significant changes in morphology when differentiated using STEMdiff™ Hepatic ODM. Organoids are ready for downstream functional analyses and characterization on day 17.

5.3.1 Differentiation Protocol Optimization Guidelines

The following protocol parameters should be optimized for every cell line and assay of interest; recommended ranges for preliminary testing in 24-well plates are provided.

PARAMETER	RECOMMENDATION	NOTES
Fragment seeding density per well for differentiation cultures	1000 - 2500 fragments per well	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.
Number of wells to be seeded for differentiation assays	2 - 6 wells per cell line per differentiation	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.
Duration of culture in complete STEMdiff™ Hepatic OGM prior to differentiation using STEMdiff™ Hepatic ODM	Culture in complete OGM for 3 - 7 days.	Organoids should be differentiated once they have expanded, but before they begin to collapse or deflate.
Duration of differentiation in complete STEMdiff™ Hepatic ODM and harvest time point	Harvest timepoints: 6 - 15 days after first ODM feed	Optimal harvest time points can vary based on analyses to be performed. Enzymatic activity or secretory functions can increase with prolonged culture in STEMdiff™ Hepatic ODM, while harvests at earlier timepoints will yield more cellular material for intracellular protein- and nucleic acid-based analyses.

TECHNICAL MANUAL

Establishment, Growth, and Differentiation of Human Pluripotent Stem Cell-Derived Hepatic Organoids Using STEMdiff™ Hepatic Organoid Media

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