Data Sheet

# 3dGRO® Human iPSC-derived Liver Organoid Progenitors

**SCC572**

**Pack Size: ≥1500 viable organoids/vial**

**Store in Liquid Nitrogen.**

FOR RESEARCH USE ONLY

**Not for use in diagnostic procedures. Not for Human or Animal Consumption.**

## Background

3dGRO® Human iPSC-derived Liver Organoid Progenitors are derived from integration-free human induced pluripotent stem cells (iPSC, SCC271) reprogrammed using Simplicon® RNA Reprogramming Technology (SCR550). Human iPS cells were differentiated using a defined, multi-step differentiation protocol progressing from definitive endoderm to hepatic endoderm, ultimately yielding liver progenitor organoids (LPOs). The resulting organoids are self-organizing, polarized 3D structures capable of long-term expansion and cryopreservation. They express key liver progenitor markers including HNF4a, SOX17, FOXA2 and SOX9, and display proper apical and basolateral polarization, indicated by E-cadherin and ZO1 expression.

For routine maintenance, 3dGRO® human iPSC-derived liver organoid progenitors can be passaged every 8–10 days using 3dGRO® Human Liver Organoid Progenitor Expansion Medium (SCM313). When cultured in differentiation medium for 14 days, these progenitor organoids can further mature into functional liver organoids containing both hepatocytes and cholangiocytes.

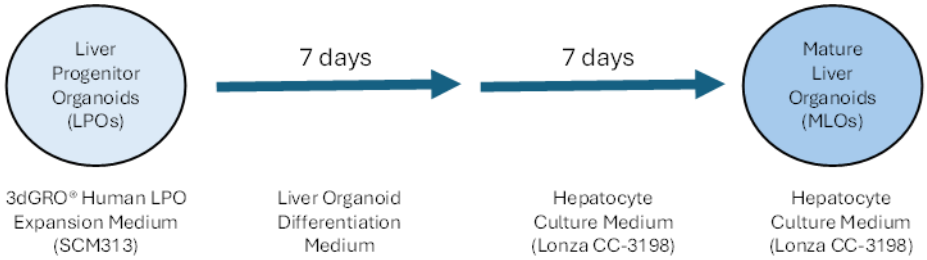


**Figure 1:** Differentiation and maintenance workflow from liver progenitor organoids (LPOs) to mature liver organoids (MLOs). This diagram illustrates the two-phase differentiation process for generating mature liver organoids (MLOs) from liver progenitor organoids (LPOs). LPOs can be cultured and maintained long-term using 3dGRO® Human Liver Progenitor Organoid Expansion Medium (SCM313). To initiate maturation, LPOs are cultured for 7 days in Liver Organoid Differentiation Medium, followed by an additional 7-day culture in Hepatocyte Culture Medium (Lonza, CC-3198), resulting in functional MLOs containing hepatocytes and cholangiocytes. Mature liver organoids can be maintained for up to 1 month in Hepatocyte Culture Medium (Lonza, CC-3198).

## Materials Provided

- 3dGRO® Human iPSC-derived Liver Organoid Progenitors (SCC572).
- One (1) vial containing ≥1500 viable organoids/vial. Store in liquid nitrogen.

## Quality Control Testing

- Viability: ≥1500 viable organoids/vial.
- Organoid Growth: Pass.
- Negative for infectious diseases by a Human Essential CLEAR panel by Charles River Animal Diagnostic Services.
- Verified to be of human origin and negative for mouse, rat, Chinese hamster, Golden Syrian hamster, and non-human primate interspecies contamination, as assessed by a Contamination Clear panel by Charles River Animal Diagnostic Services.
- Mycoplasma Contamination: Negative.
- STR Profile: Pass.

## Storage and Handling

3dGRO® Human iPSC-derived Liver Progenitor Organoids should be stored in liquid nitrogen.

## Materials Required (Not provided)

**Table 1**: Materials required but not provided.

| Products | Supplier Catalog Number | Notes |
|---|---|---|
| Corning Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix | CLS356231 | Always thaw and maintain on ice. Make 1 mL aliquots and store at −20 °C. DO NOT thaw at room temperature or at 37 °C. |
| Basal Plus Medium | | Basal Plus Medium is used for thawing, washing and passaging LPOs. Composition: DMEM/F12 PLUS Basal Medium (SCM162) containing 5 mL penicillin-streptomycin (100X), 5 mL Ala-Gln (100X) and 5 mL HEPES (1 M). |
| 3dGRO® Human Liver Progenitor Organoid Expansion Medium | SCM313 | Expansion Medium for Liver Progenitor Organoids.<br>Thaw the medium overnight at 2–8 °C. For expedited use, thaw in an ice bucket containing room temperature water. Do not thaw at 37 °C. Gently swirl the bottle to ensure thorough mixing, as components may have settled during storage. Visually inspect to confirm the medium is uniform in color with no visible gradients. Before use, allow the medium to reach room temperature naturally. Do not warm at 37 °C. Store unused medium at 2–8 °C. |
| Liver Organoid Differentiation Medium | See recipe in Table 2 | Used for the initial 7-day phase to induce differentiation of liver progenitor organoids (LPOs) into mature liver organoids. This is followed by an additional 7-day culture period in Hepatocyte Culture Medium (Lonza, CC-3198) to support further hepatic differentiation. See Figure 1. |

| | | |
|---|---|---|
| HCM™ Hepatocyte Culture Medium Bulletkit® | Lonza, CC-3198 | Used during the second 7-day phase of liver progenitor organoids (LPO) differentiation, following an initial 7-day culture in Liver Organoid Differentiation Medium. This medium supports further maturation into functional liver organoids and can also be used to maintain mature organoids for up to one month. See Figure 1. |
| DMEM/F-12 PLUS Basal Media | SCM162 | |
| DMEM-High Glucose Medium | SLM-241 | |
| 1 M HEPES Solution | H0887 | |
| Penicillin-Streptomycin (100X) | P0781 | |
| Ala-Gln, 200 mM Solution | G8541 | |
| ROCK Inhibitor, Y-27632 | SCM075 | Make 10 mM or 1000X stock by reconstituting 5 mg with 1.47 mL sterile water. Aliquot and store long-term at −20 °C. |
| Greiner CELLSTAR® 24-well tissue culture treated plates | M8812 | Pre-warmed at 37 °C for at least an hour before plating the organoids. This will help the polymerization and spreading of the Matrigel® dome upon plating. |
| 0.25% Trypsin-EDTA solution | T4049 | Aliquot in 5 mL volume, store at −20 °C. Thaw and pre-warmed to 37 °C before use. |
| CryoStor® CS10 Cell cryopreservation media | C2874 | |
| Corning® Cell Recovery Solution | CLS354253 | |
| 1X D-PBS w/o $Ca^{2+}$ and $Mg^{2+}$ | D8537 | |
| Fetal Bovine Serum | ES-009-B | |
| Sterile Freezing vials 2.0 mL | V9380 | |
| 4% Paraformaldehyde solution in PBS | Electron Microscopy Sciences 1578100 | |
| Primary & Secondary Antibody | User defined | |
| Triton® X-100 | T9284 | |

| | | |
|---|---|---|
| Normal Donkey Serum, Sterile | D9663 | |
| DAPI | D9542 | |
| Greiner Sensoplate™ glass bottom multiwell plates (96-Well) | M4187 | |
| Corning® Costar 96-Well Black Polystyrene Microplate | CLS3904 | Tissue culture treated for culturing mature liver organoids for albumin, urea, ALT, AST and GST assays. |
| Corning® Costar 96-Well White Polystyrene Microplate | CLS3903 | Tissue culture treated for culturing mature liver organoids for CYP450 assays. |
| Human ALB/Serum albumin ELISA Kit | RAB0603 | |
| Urea Assay Kit III | MAK471 | |
| Alanine Aminotransferase (ALT) Activity Assay Kit | MAK571 | |
| Aspartate Transaminase (AST) Assay Kit | MAK467 | |
| Glutathione S-Transferase (GST) Assay Kit | MAK453 | |
| CellTiter-Fluor™ Cell Viability Assay | Promega G6080 | |
| CellTiter-Glo® 3D Cell Viability Assay | Promega G9681 | |
| P450-Glo™ CYP1A2 Induction/Inhibition Assay | Promega V8421 | |
| P450-Glo™ CYP2C9 Assay | Promega V8791 | |
| P450-Glo™ CYP3A4 Assay with Luciferin-IPA | Promega V9002 | |

## Protocols and Representative Data

### Thawing of Liver Progenitor Organoids into 24-well plates

**Important notes before starting:**

- The term "domes" refers to organoids encapsulated in 3D extracellular matrices such as Matrigel® or collagen.
- It is highly recommended to culture organoids in medium supplemented with penicillin-streptomycin to prevent bacterial contamination during extended culture. Alternatively, Primocin® (InvivoGen, ant-pm-05) may be used to protect against bacterial, fungal and mycoplasma contamination.
- Each vial contains approximately 1,500–2,000 liver progenitor organoids (LPO); sufficient for 12 domes in a 24-well plate (125–160 organoids per dome).

1. Prior to thawing LPO, thaw sufficient GFR Matrigel® on ice for 12 domes at 50 µL per dome, plus a 5% overage (total: 630 µL).

   **Note:** GFR Matrigel® polymerizes quickly at room temperature. Always keep it on ice post thaw.
2. Pre-warm a sterile, tissue culture-treated 24-well plate in a 37 °C incubator for 1-2 hours before use. Label the plate with sample name, date, and passage number.
3. Prepare Basal Plus Medium: In 500 mL of DMEM/F-12 PLUS Basal Medium (SCM162), add 5 mL penicillin-streptomycin (100X), 5 mL Ala-Gln (100X) and 5 mL of 1 M HEPES. Store at 2-8 °C for up to 6 months. The Basal Plus Medium is used for washing, passaging, and thawing LPO. Pre-warm the medium to room temperature for 15 minutes before use.
4. Rapidly thaw the frozen vial of LPOs in a 37 °C bath. Immediately transfer contents to a 15 mL conical tube containing 9 mL of pre-warmed Basal Plus Medium.

   **Note**: Handle Gently. Pipetting at this point should be as gentle as possible as organoids are very fragile at this stage.
5. Rinse the cryovial with 1 mL Basal Plus Medium and add to the 15 mL conical tube.
6. Centrifuge the 15 mL tube at 350 x *g* for 5 min at 25 °C.
7. Carefully aspirate and discard the supernatant. Use a micropipette to remove any residual supernatant.
8. Remove the pre-warmed 24-well tissue culture plate from the incubator.
9. Gently resuspend the organoid pellet in 630 µL GFR Matrigel®. Avoid creating air bubbles.

   **Note**: When handling GFR Matrigel®, work as quickly as possible as GFR Matrigel® solidifies at room temperature.
10. Dispense 50 µL Matrigel®/organoids suspension per well into 12-wells of the 24-Well plate. Let the Matrigel® domes set at room temperature for 2 min. Transfer the plate to a 37 °C, 5% $CO_2$ incubator for 20 min to solidify.
11. Carefully add 650 µL of 3dGRO® Human Liver Progenitor Organoid Expansion Medium (SCM313) to each well by dispensing along the wall to avoid disturbing the dome.
12. Incubate at 37 °C, 5% $CO_2$ in a humidified incubator overnight.
13. Replace medium every 3-4 days or if the color changes to yellow.

    **Note:** LPOs are typically ready for passage after 8-14 days. Post thaw recovery may be delayed if stored at −80 °C upon receipt.
14. Observe domes under the microscope and capture images on days 0, 2, 4, 8 and the day of passage. Figure 2 shows the density and morphology on Days 0 and 8. Figure 3 displays various LPO morphologies at Day 8.
15. Passaging LPOs:
    - Passage LPOs 10-14 days post thaw.
    - After recovery, passage every 8-10 days using:
        - Trypsinization Method: split 1:8-1:10
        - Fragmentation Method: split 1:4

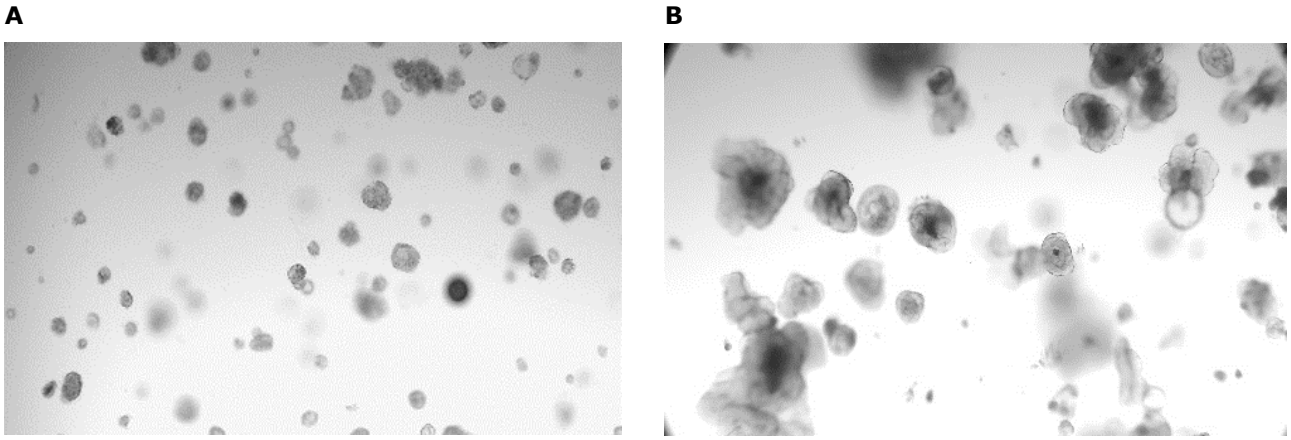


**Figure 2: A.** Morphology and density of LPO at day 0, **B.** and day 8 post thawed using protocol described. Image taken at 4X magnification**.**

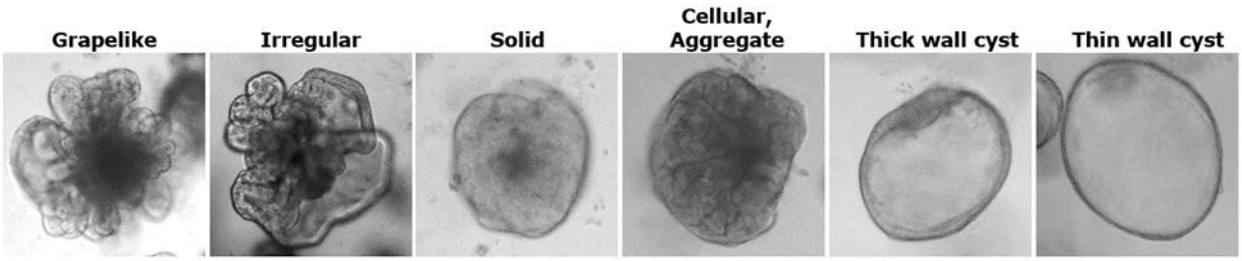


**Figure 3**: Diversity in LPO morphologies. LPOs can appear in different sizes, densities, and structures. Pictures taken at 10X magnification.

## Trypsinization-Based Passaging of Liver Progenitor Organoids

**Important notes before starting:**

Ensure that the organoids are healthy (with minimal cell death) and in the exponential growth phase before passaging using this method. Advantages of passaging liver progenitor organoids using the trypsinization method:

- Each single cell or small cell cluster gives rise to one organoid, allowing the number of organoids to be estimated during seeding.
- Organoid size is more uniform, making them suitable for biobanking and for performing functional assays following differentiation.

1. Prewarm the 24-well plates (for 1 hour), the Basal Plus Medium, DMEM with 10% FBS, 0.25% Trypsin EDTA (for 15 min) and 3dGRO® Human Liver Progenitor Organoid Expansion Medium (SCM313) at room temperature.
1. Prior to passaging the LPOs, thaw enough GFR Matrigel® vials (1 hour before).

   **Note:** GFR Matrigel® vials should be thawed on ice on the day of use or overnight on ice. GFR Matrigel® vials should always be kept on ice during use.
2. Remove media from the wells. Wash Matrigel® domes with 500 µL DPBS ($Ca^{2+}$ and $Mg^{2+}$ free, room temperature). Aspirate the D-PBS.
3. Add 450 µL of 0.25% Trypsin EDTA to each well. Using a P1000 pipette, pipette up and down vigorously 5-6 times to release the organoids from the Matrigel® dome. This step facilitates the trypsinization of the organoids.

   **Note:** Do not digest more than 12 wells from a 24-well plate per batch to avoid over-digestion. Excessive exposure to trypsin may reduce the viability of LPOs post passaging.
4. Incubate the plates at 37 °C for 9 min.
5. Remove plate from the 37 °C incubator and pipette each well up and down 5-6 times using a P1000 pipette to dissociate the organoids into single cells or small cell cluster. Add 1 mL of DMEM containing 10% FBS to each well to neutralize the trypsin. Transfer the contents of each well into a 15 mL tubes.

   **Note:** If all 12 wells contain organoids, use two 15 mL tubes to accommodate the total volume.
6. Centrifuge at 350 x *g* for 5 min at 4 °C. Aspirate the supernatant; add 10 mL of Basal Plus Medium to wash away the FBS.
7. Centrifuge again at 350 x *g* for 5 min at 4 °C. Remove all the supernatant.
8. Count cells or cell clusters using a cell counter. Calculate the number needed to seed 1500 cells or cell clusters per well.
9. Resuspend gently the calculated number of cells or cell clusters in Matrigel®. Plate 50 µL Matrigel® dome into each well of a 24-well plate.

   **Example Calculation:** To seed 1500 cells or cell clusters per well across an entire 24-well plate, 36,000 cells are required. Resuspend the 36,000 cells in 1.2 mL of Matrigel® with an additional 5% overage. Mix thoroughly and dispense 50 µL of the mixture into each well.
10. Transfer the plate to a 37 °C, 5% $CO_2$ incubator for 20 min to allow the Matrigel® to polymerize.
11. Gently add 650 µL of 3dGRO® Human Liver Progenitor Organoid Expansion Medium (SCM313) to each well. To avoid disturbing the domes, dispense the medium against the side of each well.
12. Replace the medium every 3–4 days, or sooner if the medium turns yellow.
13. Monitor the domes under a microscope and capture images on Days 0, 2, 4, 6 and the day of passaging for documentation. Figure 4 shows the morphology of LPOs on various days following passaging via the trypsinization method.
14. For biobanking, it is recommended to cryopreserve organoids on Days 6–7, when they are in the exponential growth phase, to ensure optimal recovery after thawing. For each biobank vial, pool organoids from approximately 2 wells (about 1000–1200 LPOs per well), resulting in a total of ~2000-2400 LPOs per vial.

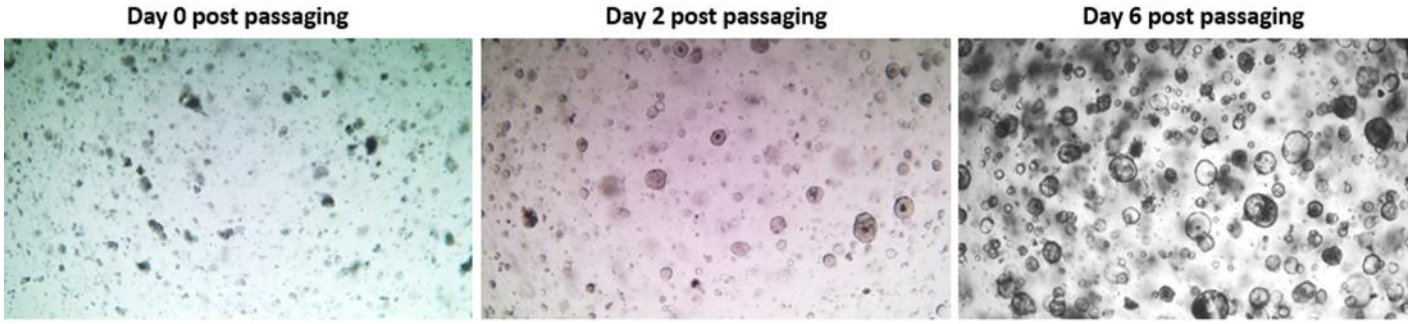


**Figure 4**: Morphology of LPOs passaged using the trypsinization method at **A.** Day 0, **B.** Day 2 and **C.** Day 6.

## Fragmentation-Based Passaging of Liver Progenitor Organoids.

Fragmentation-based passaging is recommended for the routine culture of liver progenitor organoids and for applications requiring their further differentiation into fully mature liver organoids.

1. Prewarm 24-well plates at 37 °C for 1 hour. Thaw enough vials of Matrigel® on ice for 1 hour and keep the Basal Plus Medium chilled on ice.
2. Remove the media from the wells. Add 500 µL of ice-cold Basal Plus Medium to each well of the 24-well plate containing organoids. Use a P1000 pipette to scrape and dislodge the Matrigel® dome containing the organoids, then transfer the content of each well into a 15-mL tube. Wash any remaining Matrigel® dome in the well with 100 µL of ice-cold Basal Plus Medium and add this wash to the corresponding 15 mL tube. Bring the volume in each tube up to 10 mL with the Basal Plus Medium. Keep the tubes on ice while processing the next plate.
3. Centrifuge the tubes at 350 x *g* for 5 min at 4 °C. Carefully remove the supernatant, leaving approximately 2-2.5 mL in the tube.
4. Using a P1000 pipette set to at least 900 µL, pipette the suspension up and down vigorously 10 times to break large organoids into small fragments or to dislodge small organoids from the Matrigel®.
5. Add 8 mL of ice-cold Basal Plus Medium to each tube.
6. Centrifuge again at 350 X *g* for 5 min at 4 °C. Carefully remove all the supernatant including the transparent Matrigel® that resides above the cell pellet.

   **Note**: To avoid aspirating the pellet containing organoids, it is recommended to attach a P200 pipette tip to the aspirating pipette during this step.
7. Gently resuspend the pellet in the calculated amount of Matrigel® according to the desired split ratio and number of domes. Plate 50 µL Matrigel® domes per well in a 24-well plate.
8. Transfer the plate to a 37 °C, 5% $CO_2$ incubator for 20 min to allow Matrigel® polymerization.
9. Add 650 µL of 3dGRO® Human Liver Progenitor Organoid Expansion Medium (SCM313) to each well. Incubate the plate at 37 °C in a 5% $CO_2$ incubator.
10. Change the medium every 3-4 days or whenever the medium turns yellow. This color change often occurs when cultures reach 80-100% confluency and can happen within 1-2 days.
11. Recommended split ratios:
    - 1:4 to 1:5 if confluency before passaging is 80-90%.
    - 1:2 to 1:3 if confluency before passaging is 50-70%.
12. Passaging is generally performed 8-10 days after plating, depending on the initial split ratio and confluency.
13. Observe the domes under a microscope and capture images on Days 0, 2, 4, 6 and the day of passaging for documentation. Figure 5 shows the morphology of LPOs passaged using the fragmentation method.

    **Note:** Smaller, more compact organoids (150-250 µm in diameter) may not fragment efficiently using this method as compared to larger organoids. However, the fragmentation method will promote their growth into larger and healthier organoids.

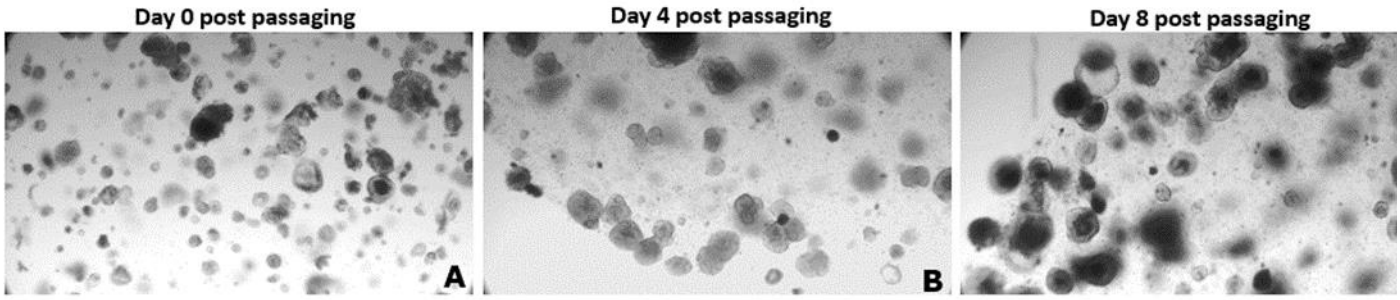


**Figure 5**: Morphology of LPOs passaged using the fragmentation method at (**A**) Day 0, (**B**) Day 4 and (**C**) Day 8

## Cryopreservation of Liver Progenitor Organoids

**Important notes before starting:**

It is recommended to cryopreserve LPOs during their exponential growth phase, typically around Days 6-7 following passaging using the trypsinization method. Figure 4 (C) illustrate the organoid density and size of the LPOs at the time of cryopreservation for biobanking.

1. Warm CryoStor® CS10 cell cryopreservation medium to room temperature.
2. Remove all medium from the plate. Add 4 mL of ice-cold Basal Plus Medium to each well and gently dislodge the Matrigel® domes using a P1000 pipette. Transfer the resulting LPO suspension into a pre-chilled 50 mL conical tube. Pool 12 wells (each containing 50 µL domes with approximately 1,000-1,200 LPOs per dome into a single 50 mL tube.
3. Centrifuge the tubes at 450 X *g* for 5 min at 4 °C.
4. Carefully aspirate the supernatant, leaving approximately 2 mL behind.
5. Pipette up and down 10 times to dislodge the LPOs from Matrigel®. Add 8 mL of ice-cold Basal Plus Medium to the tube.
6. Centrifuge at 350 x *g* for 8 min at 4 °C. Carefully remove as much supernatant as possible, including the Matrigel® layer that resides above the organoid pellet.
7. Add 6 mL of CryoStor® CS10 cell cryopreservation medium to the pellet (suitable for organoids collected from 12-wells). Aliquot 1 mL of this suspension into each cryovial. Each cryovial should contain approximately 2000-2400 LPO in freezing medium.
8. Place the cryovials in a Mr. Frosty® freezing container and store at −80 °C for 24 hours. Afterwards, transfer the vials to a liquid nitrogen tank for long term storage.

## Whole Mount Immunofluorescence

**Important notes before starting:**

- This protocol serves as a guide for first-time users and is optimized for *in situ* staining of organoids cultured in Matrigel® domes in glass-bottom 96-well plates. The Greiner Sensoplate™ Glass Bottom Multi 96-Well plate is recommended; however other imaging-compatible glass-bottom 96-Well plates may also be used.
- Once familiar with the procedure, users may adapt the protocol for whole-mount staining in other culture formats.
- Fixation with 4% paraformaldehyde partially dissolves the Matrigel® so extreme care must be taken during all pipetting and buffer exchange steps to avoid organoid loss or damage.
- Normal donkey serum is used in the blocking buffer but may be substituted depending on the host species of the secondary antibody. For instance, if using goat anti-mouse secondary antibodies, replace donkey serum with normal goat serum.
- Gentle rocking or shaking is optional and not required for all staining steps.

1. Pre-warm 4% Paraformaldehyde (PFA) to room temperature.
2. Aspirate the culture medium from each well containing the organoid domes. Wash each well once with 200 µL of 1X PBS.
3. Aspirate the PBS and add 100 µL of pre-warmed 4% PFA to each well. Fix organoids for 30 min at room temperature.
4. Carefully remove the 4% PFA and wash three times with 1X PBS. Organoids can be stored in PBS at 4 °C for up to a month before staining.
5. When ready to proceed, add 100 µL of Blocking Buffer per well (5% normal donkey serum + 0.3% Triton® X-100 in 1X PBS). Incubate for 1–2 hours at room temperature.
6. Prepare primary or directly conjugated antibodies in Blocking Buffer (100 µL per well).
7. Remove Blocking Buffer and add 100 µL of the primary antibody solution to each well. Incubate overnight at 4 °C.
8. The next day, wash 3-5 times with Wash Buffer (0.1% Triton® in 1X PBS) for 5-10 minutes per wash.

   **Tip**: If background staining is an issue, increasing the number of washes may help reduce non-specific binding.
9. Prepare secondary antibodies in Wash Buffer (100 µL per well).
10. Add the secondary antibody solution and incubate for 2-4 hours at room temperature.
11. Remove the secondary antibodies and wash 3-5 times with Wash Buffer (5-10 minutes each wash).
12. Counterstain with DAPI (5 mg/mL in Wash Buffer) for 10-15 minutes.
13. Wash 2-3 times with Wash Buffer.
14. Replace the final wash with 1X PBS. Organoids are now ready for imaging using a confocal microscope or high-content imaging system. Organoids can be stored in PBS at 4 °C for up to one week. For long term storage, it is recommended to mount with antifade reagents.

## Harvesting Organoids for qRT-PCR, ELISA, or Western Blot

1. Remove the culture medium from each well. Wash the Matrigel® domes with 500 µL of calcium and magnesium free DPBS (at room temperature), then carefully aspirate and discard the DPBS.
2. Add 500 µL of Corning Cell Recovery Solution to each well containing a 50 µL Matrigel® dome with organoids (maintaining a 1:10 ratio of Matrigel® to Cell Recovery Solution). Gently scrape the Matrigel® dome using a P1000 pipette and transfer the organoid-Matrigel®-Cell Recovery Solution mixture into a 1.5 mL microcentrifuge tube.

   **Note**: Organoids from up to 3-wells of a 24-well plate can be pooled into a single 1.5 mL microcentrifuge tube.
3. Place the tube with organoids on ice for 1 hour. Invert the tube 4-5 times every 10-15 min to facilitate Matrigel® digestion.
4. Centrifuge the tube at 10,000 x *g* for 1 min at 4 °C. Carefully remove the supernatant without disturbing the pellet. The harvested organoids are now ready RNA extraction (qRT-PCR) or cell lysate preparation for Western blot or ELISA analysis.

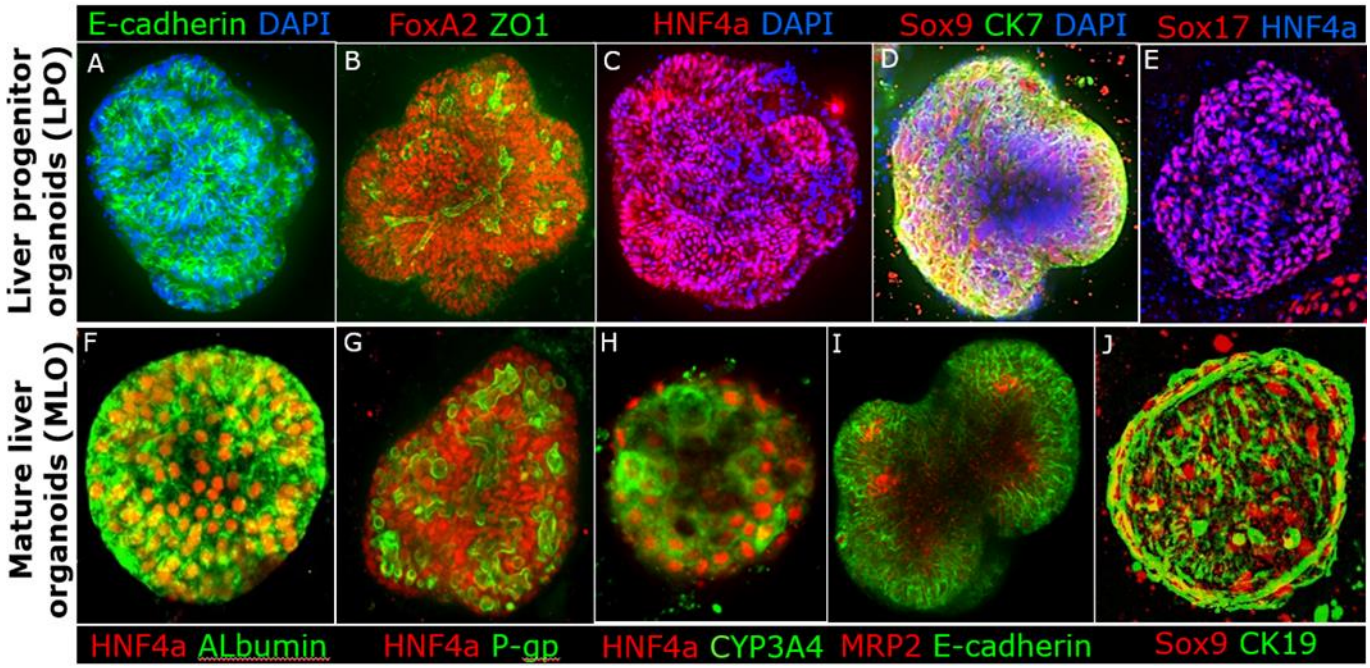


**Figure 6: A-E**. Whole-mount immunofluorescence staining of liver progenitor organoids (LPO) demonstrates that **A.** the organoids exhibit epithelial polarity, as indicated by expression of E-Cadherin (Biolegend, 324110) and **B.** ZO1 (Thermo Fisher, 33-9100). **B.** The LPOs also express key liver progenitor markers FoxA2 (WH0003170M1), **C.** HNF4a (Cell Signaling Technology, 3113), **D.** Sox9 (AB5535) and **E.** Sox17 (R&D Systems, AF1924). **F-I.** Co-expression of Sox17 and HNF4a indicates that these organoids are bipotential and capable of differentiating into both hepatocytes and **J.** cholangiocytes.

Upon differentiation into mature liver organoids (MLO), **F.** cells express markers of mature and functional hepatocyte including albumin (Thermo Fisher, PA5-89332), **H.** CYP3A4 (Thermo Fisher, MA517064) and **H.** drug transporters P-gp (Thermo Fisher, MA5-13854) and **I.** MRP2 (Cell Signaling Technology, 4446). **J.** Mature liver organoids also contain cholangiocytes, which express Sox9 and CK19 (MAB3238).

## Preparation of Liver Organoid Differentiation Medium

**Table 2:** To prepare 50 mL of Liver Organoid Differentiation Medium, combine the following components:

| Liver Organoid Differentiation Medium components | Volume (µL) | Stock concentration | Final concentration |
|---|---|---|---|
| DMEM/F-12 PLuS Basal media | 41000 | | |
| Penicillin-Streptomycin (100Xx) | 500 | 100 X | 1X |
| Ala-Gln solution (100x) | 500 | 100 X | 1X |
| HEPES Solution (100x) | 500 | 1 M | 1 mM |
| N2 supplement (100x) | 500 | 100 X | 1X |
| N-Acetylcysteine, prepared as 500 mM solution in water | 100 | 500mM | 1 mM |
| B27 Supplement (50X) | 1000 | 50X | 1X |
| hEGF, reconstituted to 100 µg/mL in 1X PBS with 0.1% BSA | 25 | 100 µg/mL | 50 ng/mL |
| HGF, reconstituted to 100 µg/mL in 1X PBS with 0.1% BSA | 12.5 | 100 µg/mL | 25 ng/mL |
| BMP7, reconstituted to 100 µg/mL in 1X PBS with 0.1% BSA | 12.5 | 100 µg/mL | 25 ng/mL |
| FGF19, reconstituted to 100 µg/mL in 1X PBS with 0.1% BSA | 50 | 100 µg/mL | 100 ng/mL |
| DAPT, reconstituted to 10 mM in DMSO | 50 | 10 mM | 10 µM |
| A83-01, reconstituted to 10 mM in DMSO | 2.5 | 10 mM | 0.5 µM |
| [Leµ$^{15}$]-Gastrin I human, reconstituted to 100 µM in 1X PBS with 0.1% BSA | 5 | 100 mM | 10 nM |
| Dexamethasone, reconstituted to 10 mM in DMSO | 15 | 10 mM | 3 µM |

All preparations are conducted under sterile conditions within a BSC hood. Store the prepared medium at 4 °C for up to 4 weeks.

**Note:** Discard any unused medium after 30 days.

1. All growth factors must be prepared in DPBS-B (1X PBS with 0.1% BSA).

   **Note:** To make 0.1% BSA solution, dilute sterile 10% BSA Stock Solution (A8806) in 1X PBS (TMS-012) and filter sterilize before use.

2. **A83-01 (TGF-beta/Smad Inhibitor, Mol. Wt. 421.52 g/mol)**

   Dissolve 25 mg of A83-01 in 5.9 mL of sterile DMSO to prepare a 10 mM stock solution. Aliquot 10 µL portions and store at −20 °C for up to 1 year.

   **Important:** Avoid repeated freeze-thaw cycles to preserve stability and activity.

3. **Dexamethasone (Mol. Wt. 392.46 g/mol)**

   Dissolve 25 mg of Dexamethasone in 6.37 mL of sterile DMSO to make a 10 mM stock solution. Aliquot 15 µL portions and store at −20 °C for up to 1 year.

   **Important:** Do not reuse thawed aliquots. Avoid repeated freeze-thaw cycles.

4. **[Leu$^{15}$]-Gastrin I Human (Mol. Wt. 2080.16 g/mol)**

   Dissolve 25 mg of [Leu$^{15}$]-Gastrin I human in 2.4 mL of sterile 1X PBS containing 0.1% BSA to prepare a 100 µM stock solution. Aliquot 10 µL portions and store at −20 °C for up to 1 year.

   **Important:** Do not reuse thawed aliquots. Avoid repeated freeze-thaw cycles.

### Differentiation of LPOs into Mature Liver Organoids

1. **Differentiation Protocol**

   Liver progenitor organoids (LPOs) can be differentiated into mature liver organoids by culturing in Liver Organoid Differentiation Medium (see Table 2 for composition) for 7 days. This is followed by a 7-day maintenance phase in Hepatocyte Culture Medium (HCM™ Hepatocyte Culture Medium BulletKit®, Lonza, CC-3198).

2. **Timing and Seeding Density**

   Initiate differentiation 10 days post-passaging of LPOs using the trypsinization method. Recommended seeding densities:

   - ~300 organoids/50 µL dome (24-well plate)
   - ~25-45 organoids/10 µL dome (96-well plate)

3. **Differentiation Notes**

   A cell death rate of approximately 5-10% is commonly observed during differentiation.

   **Important:** Mature liver organoids do not expand or proliferate and therefore cannot be passaged.

4. **Media Changes**

   Change medium every 3-4 days, or sooner if the medium appears yellowish.

5. **Post-Differentiation Maintenance**

   Mature liver organoids can be maintained in Hepatocyte Culture Medium (Lonza, CC-3198) for up to 1 month. During this time, they are suitable for downstream applications such as functional assays, DMPK studies and toxicology testing.

## Functional Characterization of Mature Liver Organoids

### Albumin secretion assay

1. **Organoid Seeding**

   Seed approximately ~25-50 LPOs (200-300 µm in diameter) per 10 µL dome into a tissue culture-treated 96-Well black plate with a flat, clear bottom (For example, Corning 96-Well Black Polystyrene Microplate, CLS3603).

2. **Differentiation**

   Differentiate LPOs into mature liver organoids following the protocol described above. See Figure 1.

3. **Albumin Detection**

   Following differentiation, collect the culture media from each well for analysis of albumin secretion using the Human ALB/Serum Albumin ELISA Kit (RAB0603). Perform the assay according to the manufacturer's instructions.

4. **Cell Viability Assay**

   Assess cell viability using the CellTiter-Fluor™ Cell Viability Assay (Promega, G6080). Follow the kit protocol carefully.

   **Note:** Use viability data to normalize albumin secretion levels measured by ELISA.

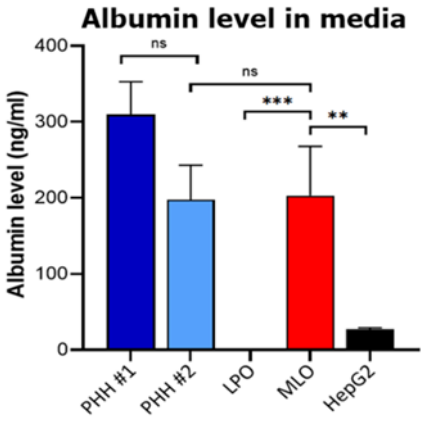


**Figure 7:** Mature liver organoids (MLO) secrete albumin at levels comparable to primary human hepatocytes (PHH), and significantly higher than liver progenitor organoids (LPO) and HepG2 cells. Two separate lots of primary human hepatocytes (PHH) were tested.

## Urea Assay

1. **Organoid Seeding**

   Seed approximately 25-50 LPOs (200-300 µM in diameter) per 10 µL dome into a tissue culture-treated 96-Well black plate with flat, clear bottom (for example, Corning 96-Well Black Polystyrene Microplate, CLS3603).

2. **Differentiation**

   Differentiate LPOs into mature liver organoids following the protocol described above. See Figure 1.

3. **Pre-treatment with Urea Precursors**

   48-hours prior to performing the urea assay, replace the culture medium with 100 µL of phenol red-free Hepatocyte Culture Medium (Lonza, CC-3198) supplemented with urea production precursors (see Table 3). Incubate for 24-hours.

   **Note:** Use only phenol red-free medium when performing the assay with the Urea Assay Kit III (MAK471).

4. **Ammonium Chloride Stimulation**

   After the 24-hour incubation, add 100 µL of Hepatocyte Culture Medium (Lonza, CC-3198) containing 3 mM ammonium chloride ($NH_4Cl$) directly to the wells without removing the existing medium. This results in a final $NH_4Cl$ concentration of 1.5 mM.

5. **Sample Collection and Urea Measurement**

   Incubate for an additional 24-hours, then collect the medium from each well. Perform the urea assay immediately using the Urea Assay Kit III (MAK471) according to the manufacturer's instructions.

6. **Cell Viability Assay**

   Assess cell viability using the CellTiter-Fluor™ Cell Viability Assay (Promega, G6080), following the kit protocol.

   **Note:** Use cell viability data to normalize urea secretion values.

**Table 3:** Recipe for Hepatocyte Culture Medium containing precursor for urea production.

| Components | Final Conc. |
|---|---|
| Hepatocyte Culture Medium (Lonza, CC-3198) | 1X |
| L-Glutamine (59202C) | 2 mM |
| D-galactose (3455) | 2.27 mM |
| L-Lactate (L7022) | 2 mM |
| Ornithine hydrochloride (W419001) | 2.5 mM |

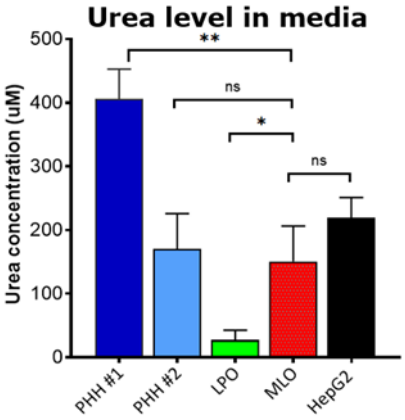


**Figure 8:** Mature liver organoids (MLO) secrete urea at levels comparable to primary hepatocytes (PHH) and HepG2 cells. Urea secretion from mature liver organoids (MLO) is significantly higher than that from liver progenitor organoids (LPO), indicating functional maturation.

## CYP1A2, CYP2C9 and CYP3A4 Activity Assays

1. **Organoid Seeding**

   Seed approximately 25-50 LPOs (200-300 µM in diameter) per 10 µL dome into a tissue culture-treated 96-well white plate with a flat, clear bottom (for example, Corning 96-Well White Polystyrene Microplate, CLS3610).

2. **Differentiation**

   Differentiate LPOs into mature liver organoids following the protocol described above. See Figure 1. Allocate sufficient wells for each cytochrome P450 (CYP) assay, including conditions for uninduced, induced and induced + inhibitory (rescue) groups.

3. **Induction of CYP Activity**

   To induce CYP activity, replace the medium with Hepatocyte Culture Medium (Lonza, CC-3198) containing specific inducers 48 hours prior to performing the assays:

   - CYP1A2: 100 µM Omeprazole
   - CYP2C9: 25 µM Rifampicin
   - CYP3A4:. 25 µM Rifampicin

4. Incubate organoids with inducer for 24-48 hours. Perform extensive washing with 1X PBS or Basal Plus Medium to remove inducer completely. To completely remove the inducers trapped in the Matrigel®, it is recommended to incubate at 37 °C for 5 minutes between washes.

5. **CYP Activity Assays**

   Measure CYP enzyme activity using the following luciferase-based assays from Promega:

   - CYP1A2: P450-Glo™ CYP1A2 Induction/Inhibition Assay (Promega, V8421)
   - CYP2C9: P450-Glo™ CYP2C9 Assay (Promega, V8791)
   - CYP3A4: P450-Glo™ CYP3A4 Assay with Luciferin-IPA (Promega, V9001)

6. Follow the manufacturer's instructions for cell-based assays.

7. **Cell Viability Assay**

   Evaluate cell viability using the CellTiter-Glo® 3D Cell Viability Assay Kit (Promega, G9681).

   **IMPORTANT**: Follow the viability protocol provided within the CYP450 assay kit and **NOT** the protocol provided in the CellTiter-Glo® 3D Cell Viability Assay Kit.

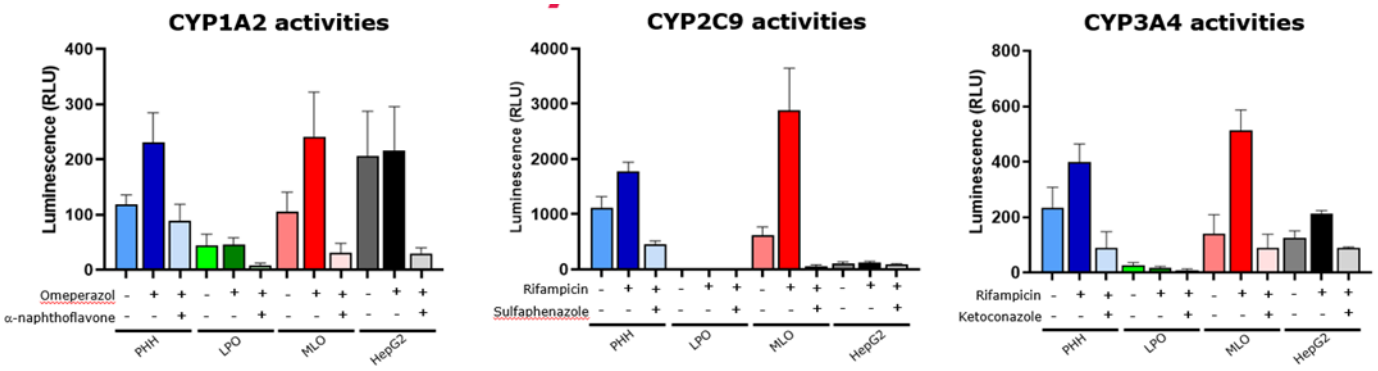


**Figure 9:** Mature liver organoids (MLO) exhibit both basal and inducible activities of key cytochrome P450 enzymes (CYP1A2, CYP2C9 and CYP3A4) at levels comparable to or exceeding those of primary human hepatocytes (PHH) and HepG2 cells. Enzyme activity can be upregulated by inducers (omeprazole and rifampicin) and suppressed by specific inhibitors (α-naphthoflavone, sulfaphenazole and ketoconazole). In contrast, liver progenitor organoids (LPO) show minimal CYP450 activity.

## ALT, AST and GST activity assay

1. **Organoid Seeding**

   Seed approximately 25-50 LPOs (200-300 µM in diameter) per 10 µL dome into a tissue culture-treated 96-Well black plate with a flat, clear bottom (for example, Corning 96-Well Black Polystyrene Microplate, CLS3603).

2. **Differentiation**

   Differentiate LPOs into mature liver organoids as described above. See Figure 1.

3. **Cell Viability Assessment**

   On the day of the assay, perform cell viability measurements using the CellTiter-Fluor™ Cell Viability Assay (Promega, G6080) before the ALT, AST and GST assays. Follow the manufacturer's instructions.

   **Note:** Viability data will be used to normalize ALT, AST and GST enzyme activity levels.

4. **Membrane Permeabilization (Optional)**

   Following the viability assay, treat organoids with 0.2% Triton® X-100 in Basal Plus Medium for 15-20 min at 37 °C to permeabilize cell membranes and release ALT, AST, and GST enzymes into the medium.

   **Important:** This step is applicable only when enzyme activity is measured without drug treatment. If assessing the impact of drug treatments on enzyme activity, do not perform the Triton® X-100 permeabilization step.

5. **Enzyme Activity Assays**

   Measure ALT, AST and GST activities using the following assay kits.

   - ALT: Alanine Aminotransferase Activity Assay (MAK052)
   - AST: Aspartate Transaminase (AST) Assay Kit (MAK467)
   - GST: Glutathione-S-Transferase (GST) Assay Kit (CS0410)

Follow the manufacturer's protocols for each assay.

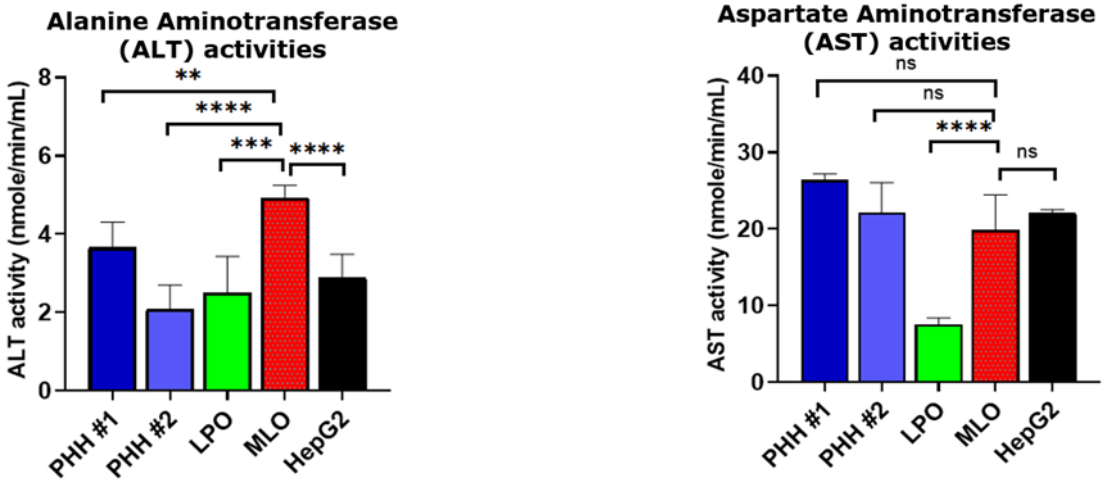


**Figure 10:** Mature liver organoids (MLO) exhibit alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities at levels comparable to those of primary hepatocytes (PHH) and HepG2 cells, supporting their functional maturity as liver organoids. Two separate lots of primary hepatocytes (PHH) were tested.

## Related Products

3dGRO® Human Liver Progenitor Organoid Expansion Medium (SCM313).

## References


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2. Mun SJ, Ryu J-S, Lee M-O, Son YS, Oh SJ, Cho H-S, Son M-Y, Kim D-S, Kim SJ, Yoo HJ, et al. 2019. Generation of expandable human pluripotent stem cell-derived hepatocyte-like liver organoids. J Hepatol. 71(5): 970-985.
3. Hu H, Gehart H, Artegiani B, LÖpez-Iglesias C, Dekkers F, Basak O, Es JV, Lopes SMCdS, Begthel H, Korving J, et al. 2018. Long-term expansion of functional mouse and human hepatocytes as 3D organoids. Cell. 175(6): 1591-1606.

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