# Culture of Human Liver Organoids

## Using Cultrex™ UltiMatrix Basement Membrane Extract

This protocol provides a procedure for subculturing normal human liver organoids. This protocol was modified from the submerged method described in Huch, M. *et al.* (2015) Cell. **160**: 299. The protocol provided below is intended to culture organoids from normal human liver tissues using Cultrex™ UltiMatrix Reduced Growth Factor (RGF) Basement Membrane Extract as a scaffold. The majority of reagents used in this protocol were sourced from the Bio-Techne brands of R&D Systems™ and Tocris Bioscience™.

**TABLE // 01**

Materials Needed for Human Liver Organoid Culture

| Product Name | Supplier | Catalog # |
|---|---|---|
| **Cultrex Organoid Harvesting Solution** | R&D Systems | 3700-100-01 |
| **Cultrex UltiMatrix Reduced Growth Factor Basement Membrane Extract** | R&D Systems | BME001-05 |
| **Advanced DMEM/F-12 Cell Culture Medium** | Thermo Fisher | 12634010 |
| **GlutaMAX™** | Thermo Fisher | 35050061 |
| **HEPES** | Tocris Bioscience | 3173 |
| **Penicillin/ Streptomycin** | Various | Various |
| **N21-MAX Supplement** | R&D Systems | AR008 |
| **N-2 MAX Supplement** | R&D Systems | AR009 |
| **N-Acetylcysteine** | Tocris Bioscience | 7874 |
| **Gastrin I (Human)** | Tocris Bioscience | 3006 |
| **Nicotinamide** | Tocris Bioscience | 4106 |
| **Y-27632 dihydrochloride (Rho Kinase inhibitor)** | Tocris Bioscience | 1254 |
| **A 83-01 (ALK5 inhibitor)** | Tocris Bioscience | 2939 |
| **Forskolin** | Tocris Bioscience | 1099 |
| **DAPT** | Tocris Bioscience | 2634 |
| **Dexamethasone** | Tocris Bioscience | 1126 |
| **Recombinant Human EGF** | R&D Systems | 236-EG |
| **Recombinant Human R-Spondin 1** | R&D Systems | 4645-RS |
| **Recombinant Human Noggin** | R&D Systems | 6057-NG |
| **Recombinant Human FGF-10** | R&D Systems | 345-FG |
| **Recombinant Human FGF-19** | R&D Systems | 969-FG |
| **Recombinant Human BMP-7** | R&D Systems | 354-BP |
| **Recombinant Human HGF** | R&D Systems | 294-HG |
| **Recombinant Human Wnt-3a** | R&D Systems | 5036-WN |

## Other Required Reagents

- Distilled (DW) or deionized water (DI)
- Phosphate buffered saline (PBS)

# Reagent Preparation

Use aseptic technique at all times during this protocol. This protocol is optimized for human liver organoids. Organoids from other tissues may have different culture requirements.

1. Thaw Cultrex UltiMatrix RGF Basement Membrane Extract (BME) on ice for four hours or overnight at 2 - 8 °C (on ice in the refrigerator).
2. Prepare Liver Organoid Initiation Medium (Table 2), Liver Organoid Expansion Medium (Table 3), and Liver Organoid Differentiation Medium (Table 4).
3. Sterile filter the media.

TABLE // 02

Human Liver Organoid Initiation Medium

| Reagent Name | [FINAL] |
|---|---|
| **Advanced DMEM/F-12 Cell Culture Medium** | NA |
| **N21-MAX Supplement** | 1X |
| **GlutaMAX™** | 2 mM |
| **HEPES** | 10 mM |
| **Penicillin/Streptomycin** | 1X |
| **N-2 MAX Supplement** | 1X |
| **Nicotinamide** | 10 mM |
| **A 83-01** | 5 µM |
| **N-Acetylcysteine** | 1.25 mM |
| **Recombinant Human FGF-10** | 100 ng/mL |
| **Recombinant Human R-Spondin 1** | 0.5 µg/mL |
| **Gastrin I Human** | 10 nM |
| **Recombinant Human BMP-7** | 25 ng/mL |
| **Recombinant Human EGF** | 50 ng/mL |
| **Recombinant Human HGF** | 25 ng/mL |
| **Recombinant Human Noggin** | 25 ng/mL |
| **Recombinant Human Wnt-3a** | 100 ng/mL |
| **Forskolin** | 10 µM |
| **Y-27632 dihydrochloride** | 10 µM |

TABLE // 03

Human Liver Organoid Expansion Medium

| Reagent Name | [FINAL] |
|---|---|
| **Advanced DMEM/F-12 Cell Culture Medium** | NA |
| **N21-MAX Supplement** | 1X |
| **GlutaMAX™** | 2 mM |
| **HEPES** | 10 mM |
| **Penicillin/Streptomycin** | 1X |
| **N-2 MAX Supplement** | 1X |
| **Nicotinamide** | 10 mM |
| **A 83-01** | 5 µM |
| **N-Acetylcysteine** | 1.25 mM |
| **Recombinant Human FGF-10** | 100 ng/mL |
| **Recombinant Human R-Spondin 1** | 0.5 µg/mL |
| **Gastrin I Human** | 10 nM |
| **Recombinant Human BMP-7** | 25 ng/mL |
| **Recombinant Human EGF** | 50 ng/mL |
| **Recombinant Human HGF** | 25 ng/mL |
| **Forskolin** | 10 µM |

TABLE // 04

Human Liver Organoid Differentiation Medium

| Reagent Name | [FINAL] |
|---|---|
| **Advanced DMEM/F-12 Cell Culture Medium** | NA |
| **N21-MAX Supplement** | 1X |
| **GlutaMAX™** | 2 mM |
| **HEPES** | 10 mM |
| **Penicillin/Streptomycin** | 1X |
| **N-2 MAX Supplement** | 1X |
| **A 83-01** | 0.5 µM |
| **Recombinant Human FGF-19** | 100 ng/mL |
| **Gastrin I Human** | 10 nM |
| **Recombinant Human BMP-7** | 25 ng/mL |
| **Recombinant Human EGF** | 50 ng/mL |
| **Recombinant Human HGF** | 25 ng/mL |
| **DAPT** | 10 µM |
| **Dexamethasone** | 30 µM |

# Methods for Expanding and Differentiating Human Liver Organoids

## Expansion

1. Prepare a suspension of isolated and dissociated human liver tissues as detailed in Huch, M. *et al.* (2015) Cell **160**: 299.
2. Resuspend the cell pellet in Cultrex UltiMatrix RGF Basement Membrane Extract and aliquot into wells by dispensing 50 µL of the Cultrex UltiMatrix RGF Basement Membrane Extract liver cell mixture in the center of each well of a 24-well plate to create domes.

   **Note:** The Cultrex UltiMatrix RGF BME domes should not touch the sides of the well.
3. Incubate the plate in the cell culture incubator for 15 minutes to polymerize the Cultrex UltiMatrix RGF Basement Membrane Extract.
4. Add the appropriate volume (~500 µL/well) of Liver Organoid Initiation Medium (Table 2).
5. Return the plate containing the organoid cultures to the cell culture incubator to promote growth.
6. After 3 days, aspirate the culture medium from each well and add fresh Liver Organoid Expansion Medium (Table 3) every other day (~500 µL/well).

   **Note:** Medium should be gently aspirated from and pipetted into the corner of the well away from the Cultrex UltiMatrix RGF BME/ organoids to prevent their disruption.
7. Passage organoids (See Passage Liver Organoids section) or start differentiation after 7-10 days.

## Differentiation

1. Following 7-10 days of expansion, aspirate the Liver Organoid Expansion Medium, wash once with PBS, then add an equal volume (~500 µL/well) of Liver Organoid Differentiation Medium (Table 4). Incubate for an additional 11-13 days.
2. The culture medium should be aspirated from each well and replaced with fresh Liver Organoid Differentiation Medium every other day.

   **Note:** Medium should be gently aspirated from and pipetted into the corner of the well away from the Cultrex UltiMatrix RGF BME/ organoids to prevent their disruption.

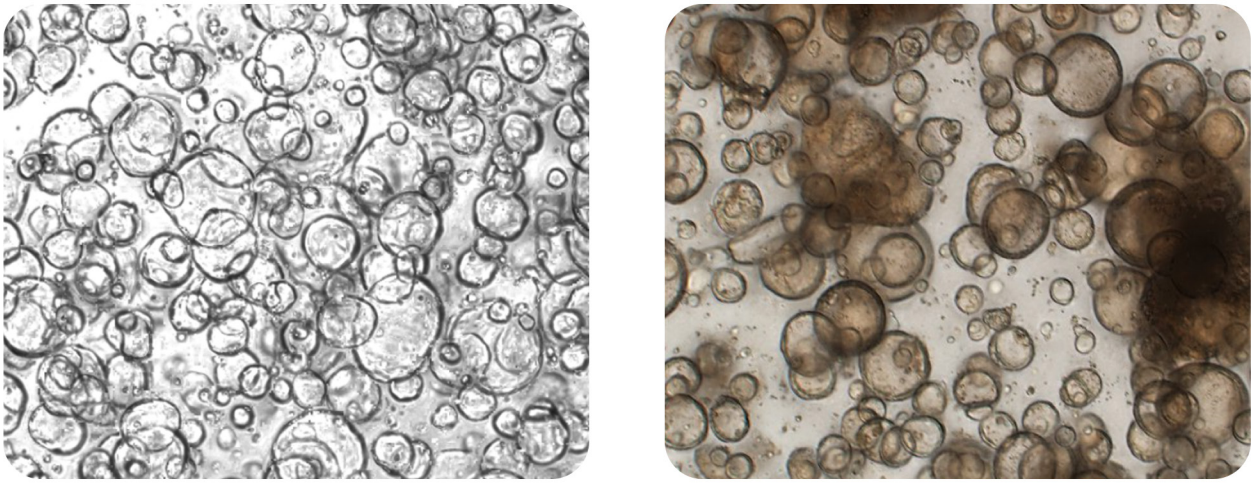

**Figure 1.** Undifferentiated and Differentiated Human Liver Organoids. Representative brightfield images of undifferentiated (left) and differentiated (right) human liver organoids that were cultured using Cultrex UltiMatrix RGF Basement Membrane Extract (R&D Systems, Catalog # BME001-05) and the reagents listed in this protocol.

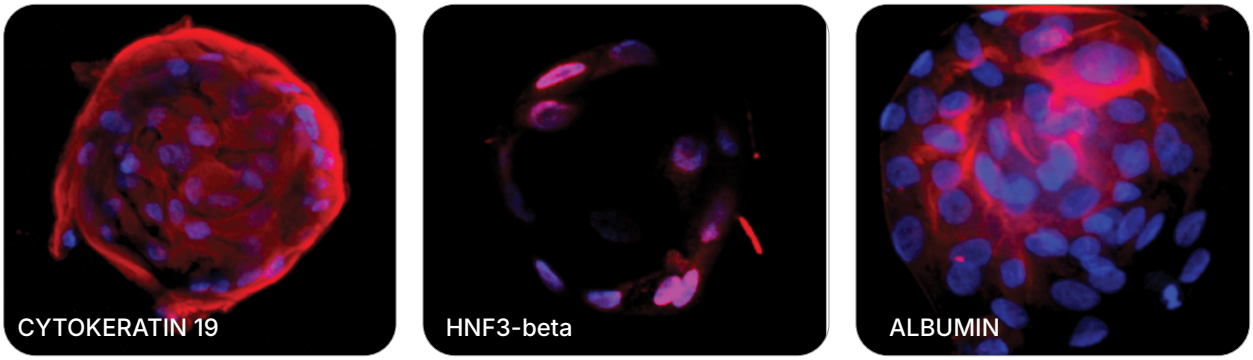


**Figure 2.** Characterization of Differentiated Human Liver Organoids. Adult stem cell-derived liver organoids were cultured using Cultrex UltiMatrix RGF Basement Membrane Extract (R&D Systems, Catalog # BME001-05) and the other reagents listed in this protocol. Differentiated human liver organoids were stained for proteins characteristic of differentiated hepatocytes using a Sheep Anti-Human Cytokeratin 19 Polyclonal Antibody (R&D Systems, Catalog # AF3506), a Goat Anti-Human HNF3-beta Polyclonal Antibody (R&D Systems, Catalog # AF2400), and a Mouse Anti-Human Serum Albumin Monoclonal Antibody (R&D Systems, Catalog # MAB1455). Organoids were counterstained with DAPI (Tocris, Catalog # 5748).

## Passaging Liver Organoids

1. View liver organoids under the microscope. Each well should contain approximately 500 organoids for optimal growth. Organoid cultures exhibiting rapid growth may be split 1:4 during passaging, while slow growing cultures may benefit from a 1:1 split. Make this determination prior to harvesting to estimate reagent needs prior to starting.

   **Note:** Organoid density is important for optimal growth; too many organoids will strain culture resources, while too few organoids lack paracrine signaling necessary to sustain growth.

2. Transfer the 24-well plate containing liver organoids from the cell culture incubator to the cell culture hood.

3. Aspirate the medium without disturbing the Cultrex UltiMatrix RGF BME-contained organoids at the bottom of the well.

4. Gently wash each well with 10 volumes of cold (2-8 °C) PBS (Table 5). Be careful not to disrupt the basement membrane matrix-containing organoids.

5. Aspirate the PBS, and add 10 volumes of cold (2-8 °C) Cultrex Organoid Harvesting Solution to each well (Table 5).

6. Incubate the plate at 2-8 °C or on ice for 30–90 minutes with moderate shaking. This incubation is complete when the basement membrane matrix dome is no longer visible at the bottom of the well and the organoids are seen floating at the bottom of the well.

   **Note:** Dislodging the dome with a cell scraper or pipet may accelerate this process.

7. Once the matrix depolymerizes, transfer the contents of the well into a tube on ice. Single wells may be transferred to a microtube while multiple domes may necessitate a 15 mL or 50 mL conical tube.

8. Centrifuge the tube at 500 x g for 5 minutes at 2-8 °C in a swinging bucket rotor to pellet the organoids. Aspirate the supernatant.

9. Wash the organoids with 10 volumes of cold (2-8 °C) PBS, and repeat centrifugation at 500 x g for 5 minutes at 2- 8 °C in a swinging bucket rotor to pellet the organoids. Aspirate the PBS. Add fresh ice-cold Liver Organoid Expansion Medium.

10. Pipet up and down three times with a serological pipette to mix the organoids.

TABLE // 05

Suggested Working Volumes of PBS and Cultrex Organoid Harvesting Solution

| Plate Type | Volume of Basement Membrane Matrix | Volume of PBS and Organoid Harvesting Solution |
|---|---|---|
| **96-well plate** | 5 µL | 50 µL |
| **48-well plate** | 25 µL | 250 µL |
| **24-well plate** | 50 µL | 500 µL |

11. Centrifuge the tube at 500 × g at room temperature for 3 minutes.

12. Aspirate the medium, but be careful not to disturb the organoid pellet.

13. Resuspend the organoids in Cultrex UltiMatrix RGF Basement Membrane Extract, and dispense 50 µL of the mixture in the center of each well of a 24-well plate to form domes. Follow the density/splitting ratios recommended in Passaging Protocol Step 1.

    **Note:** The Cultrex UltiMatrix RGF BME-contained organoids should not touch the sides of the well.

14. Incubate the plate in the cell culture incubator for 15 minutes to polymerize the Cultrex UltiMatrix RGF Basement Membrane Extract.

15. Add 500 µL of Liver Organoid Initiation Medium per well.

    **Note:** Medium should be gently pipetted into the corner of the well away from the Cultrex UltiMatrix RGF BME/organoids to prevent their disruption.

16. Return the plate containing organoid cultures to the cell culture incubator to promote organoid growth. Follow Liver Organoid Expansion Protocol.

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