

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.

EQUIPMENT

1. Cell culture incubator (37 °C, 5% CO₂)
2. Cell culture hood with laminar flow
3. Centrifuge with refrigeration and swinging bucket rotor
4. 37 °C water bath
5. Ice bucket
6. Laboratory refrigerator
7. Pipet aid and serological pipettes (5 mL)
8. Micropipettes and tips (2–200 µL)
9. Conical tubes, 10 mL and 50 mL, sterile
10. 24-well plate, tissue-culture treated, sterile
11. Vacuum pump
12. Medium filtration unit, 0.1 µm, 500 mL, sterile
13. Syringe, 50 mL, sterile
14. Syringe filter, 0.2 µm, sterile
15. Cell culture waste container

OTHER REQUIRED REAGENTS

1. Distilled (DW) or deionized water (DI)
2. Phosphate buffered saline (PBS)

MATERIALS

PRODUCT NAME	SUPPLIER	CATALOG #
Cultrex Organoid Harvesting Solution	R&D Systems	3700-100-01
Cultrex UltMatrix RGF Basement Membrane Extract	R&D Systems	BME001-05
Advanced DMEM/F-12 Cell Culture Medium		
Glutamine	Tocris Bioscience	5823
HEPES	Tocris Bioscience	3173
N21-MAX Supplement	R&D Systems	AR008
N-2 MAX Supplement	R&D Systems	AR009
N-Acetylcysteine	Tocris Bioscience	5619
Gastrin I (Human)	Tocris Bioscience	3006
Nicotinamide	Tocris Bioscience	4106
Y-27632 dihydrochloride (Rho kinase inhibitor)	Tocris Bioscience	1254
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
Forskolin	Tocris Bioscience	1099
A 83-01 (ALK5 inhibitor)	Tocris Bioscience	2939
Recombinant Human Wnt-3a	R&D Systems	5036-WN
DAPT	Tocris Bioscience	2634
Dexamethasone	Tocris Bioscience	1126

TABLE 1. Materials needed for human liver organoid culture

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 overnight 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

REAGENT NAME	[FINAL]
Advanced DMEM/F-12 Cell culture Medium	NA
N21-MAX Supplement	1X
Glutamine	2 mM
HEPES	10 mM

TABLE 2. Preparation of Human Liver Organoid Initiation Medium.

REAGENT NAME	[FINAL]
Advanced DMEM/F-12 Cell culture Medium	NA
N21-MAX Supplement	1X
Glutamine	2 mM
HEPES	10 mM
Penicillin/Streptomycin	1X
N-2 MAX Supplement	1X
Nicotinamide	10 mM
A 83-01	5 μ M
N-Acetylcysteine	1.25 mM
Recombinant Human FGF-10	100 ng/mL
Recombinant Human R-Spondin 1	0.5 μ g/mL
Gastrin I Human	10 nM
Recombinant Human BMP7	25 ng/mL
Recombinant Human EGF	50 ng/mL
Recombinant Human HGF	25 ng/mL
Forskolin	10 μ M

TABLE 3. Human Liver Organoid Expansion Medium.

REAGENT NAME	[FINAL]
Penicillin/Streptomycin	1X
N-2 MAX Supplement	1X
Nicotinamide	10 mM
A 83-01	5 μ M
N-Acetylcysteine	1.25 mM
Recombinant Human FGF-10	100 ng/mL
Recombinant Human R-Spondin 1	0.5 μ g/mL
Gastrin I Human	10 nM
Recombinant Human BMP7	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 μ M
Y-27632	10 μ M

TABLE 2. Preparation of Human Liver Organoid Initiation Medium.

REAGENT NAME	[FINAL]
Advanced DMEM/F-12 Cell culture Medium	NA
N21-MAX Supplement	1X
Glutamine	2 mM
HEPES	10 mM
Penicillin/Streptomycin	1X
N-2 MAX Supplement	1X
A 83-01	0.5 μ M
Recombinant Human FGF-19	100 ng/mL
Gastrin I Human	10 nM
Recombinant human BMP7	25 ng/mL
Recombinant Human EGF	50 ng/mL
Recombinant human HGF	25 ng/mL
DAPT	10 μ M
Dexamethasone	30 μ M

TABLE 4. Human Liver Organoid Differentiation Medium.

PROTOCOL

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 cell pellet in Cultrex UltiMatrix RGF Basement Membrane Extract and aliquot into wells and dispense 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 ($\sim 500 \mu\text{L}/\text{well}$) of Liver Organoid Initiation Medium (TABLE 1).
5. Return plate containing 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 2) every other day ($\sim 500 \mu\text{L}/\text{well}$). **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 Liver Organoid Expansion Medium, wash once PBS, then add an equal volume ($\sim 500 \mu\text{L}/\text{well}$) of Liver Organoid Differentiation Medium (Table 3). 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.*

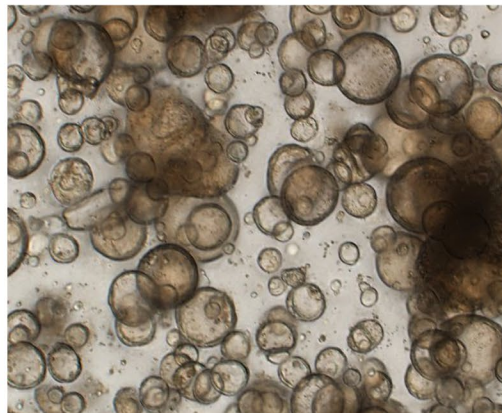
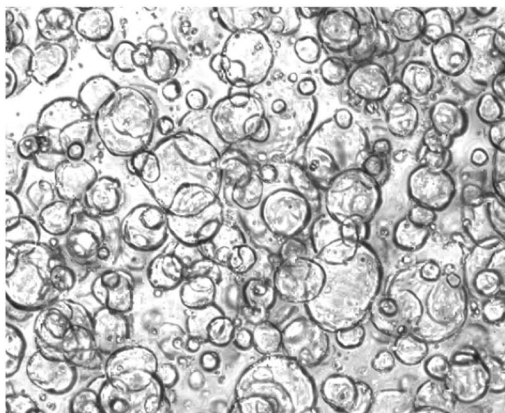


FIGURE 1. Undifferentiated (Left) and Differentiated (Right) Human Liver Organoids. Representative brightfield images of human liver organoids that were cultured using Cultrex UltiMatrix RGF BME (Catalog # BME001-05) and the reagents listed in this protocol.

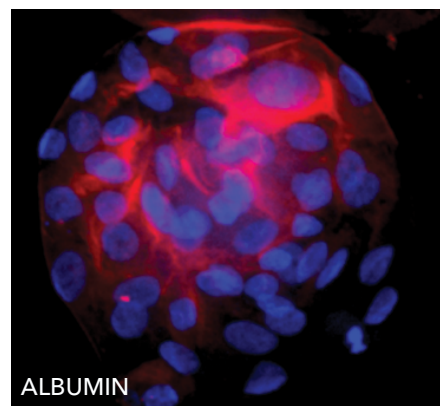
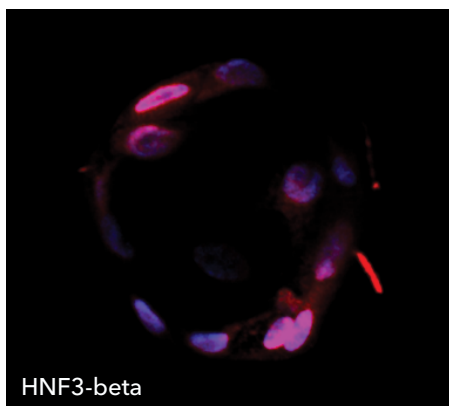
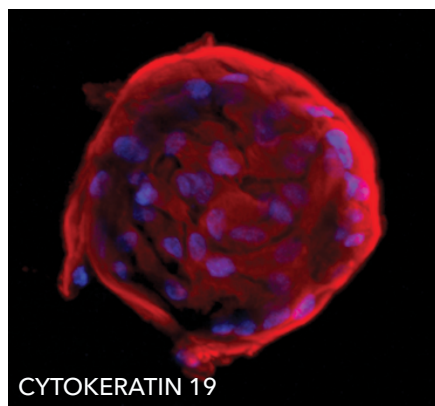


FIGURE 2. Characterization of Differentiated Human Liver Organoids. Representative images of differentiated liver expressing proteins characteristic of differentiated hepatocytes, including *Cytokeratin 19* (R&D Systems, Catalog # AF3506), *HNF3-beta* (R&D Systems, Catalog # AF2400), and *Albumin* (R&D 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 tube.
4. Gently wash each well with 10 volumes of cold (2-8 °C) PBS (TABLE 1). Be careful not to disrupt 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 1).
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 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 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.

PLATE TYPE	VOLUME OF BASE-MENT MEMBRANE MATRIX	VOLUME OF PBS AND ORGANOID HAR-VESTING SOLUTION
96-well plate	5 µL	50 µL
48-well plate	25 µL	250 µL
24-well plate	50 µL	500 µL

TABLE 1: Suggested working volumes of PBS and Cultrex Organoid Harvesting Solution.

11. Centrifuge the tube at 500 × g at room temperature for 3 minutes.
12. Aspirate medium, but be careful not to disturb the organoid pellet.
13. Resuspend 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 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 plate containing organoid cultures to the cell culture incubator to promote organoid growth. Follow Liver Organoid Expansion Protocol.

Learn more | rndsystems.com/organoids

View Our Full Cultrex UltiMatrix Protocol Video | rndsystems.com/products/cellculture