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HUMAN PRIMARY LIVER ENDOTHELIAL AND SINUSOIDAL CELLS CULTURE

PRODUCT DETAILS

Tissue	Human liver
Shipped	Cryopreserved
Storage	Liquid Nitrogen
Growth Properties	Adherent

PROCEDURE

The aim of the present method is to describe the process of thawing, culturing, cell passaging, and cryopreservation of primary human liver endothelial and sinusoidal cells.

Please read through this entire protocol before attempting this procedure.

Required and recommended reagents and consumables

- Thawing and culture protocol
  - **MNT:** NPCs Thawing Medium
  - **MEG:** Liver Endothelial and Sinusoidal Cells Growing Medium
  - **Cell culture plates**
  - **Fibronectin**
- Cell passaging
  - **MEG:** Liver Endothelial and Sinusoidal Cells Growing Medium
  - **DPBS:** Dulbecco's Phosphate Buffered Saline, without Calcium & Magnesium (DPBS)
  - **TrypLE Express**
  - **Cell culture plates**
  - **Fibronectin**
- Cell cryopreservation
  - **MEG:** Liver Endothelial and Sinusoidal Cells Growing Medium
  - **DPBS:** Dulbecco's Phosphate Buffered Saline, without Calcium & Magnesium (DPBS)
  - **TrypLE Express**
  - **MLC:** Hepatic Cells Cryopreservation Medium
  - **Cryotubes**
  - **Cryocooler**

1. Arrival of the cryopreserved cells in your facilities

Store the cryogenic liver endothelial and sinusoidal vials immediately in vapor phase nitrogen upon receipt.

2. Thawing and culture protocol for cryopreserved primary human liver endothelial and sinusoidal cells

Note: Handle gently and quickly to maintain viability. Before the thawing process, prepare a coated plate with fibronectin at 4ug/cm^2.

Thawing of primary human liver endothelial and sinusoidal cells

1. Before starting the thawing process, MNT has to be warmed at 37 °C in a water bath.

2. Remove the cryopreserved liver endothelial and sinusoidal cells from the liquid nitrogen and immediately place it in a 37 °C water bath, hold and rotate the vial gently until the contents are partially thawed.

3. Remove the vial from the water bath, wipe dry, rinse the cryovial with 70% ethanol for disinfection and transfer it into the sterile flow hood.

4. Using a pipette, gently transfer the cell suspension contained in the cryovial into a sterile 15 ml conical tube with 5 ml preheated MNT.

5. Add 1 ml of MNT to the cryovial, in order to recover the cells that have remained inside each cryovial. Then, combine it with the cells in the 15 ml conical tube to ensure that all endothelial and sinusoidal cells have been transferred.

6. Centrifuge the tube at 430g for 7 min.

7. After centrifugation, aspirate the supernatant and resuspend the cell pellet in 1-3 ml of fresh MEG by gently agitating the bottom of the tube.

8. Perform a cell count (see the next section: determination of viability and number of cells)

Determination of viability and number of cells

To determine viability and number of cells from the endothelial and sinusoidal cells' suspension in step 8, please follow these instructions:

- Carefully homogenize the cell suspension (do not vortex).
- Take 20 µl of the cell suspension and mix it in a 1.5 ml tube with 20 µl of PBS, and 20 µl of Trypan Blue solution (1/3 dilution) and keep the rest of the cell suspension on ice in the laminar flow hood until seeding.
- Gently homogenize the solution by pipetting up/down.
- Add an aliquot of 20 µl with the Trypan Blue/cell mixture into a cell counting chamber.

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- Proceed with cell observation and counting under the microscope. Dead cells will appear in blue. In order to determine viability and cell number, count the living and dead cells and use these formulas:

Viability (%) = (Live cell count/(Live cell count + dead cell count)) x 100

Total Cell number = (Viable cell count/Quadrants counted) x Dilution factor x 10^4* x Current volume(ml)

**This factor is applicable when it is used a Hemocytometer*

- After determining cell viability and total number of cells, proceed with cell seeding as follows.

Instructions for seeding human liver endothelial and sinusoidal cells:

1. After counting the cells, seed them at a cell density of 10.000-15.000 cells/ cm^2 in the desired precoated fibronectin cell culture plates with MEG.
2. Once the plates have been seeded shake vigorously in 8 form, except the 96-well plates that should NOT be shaken.
3. Insert the cells in the incubator at 37°C/5% CO_2.
4. Do not disturb the culture for at least 16 hours after seeding.

Maintenance of the human liver endothelial and sinusoidal cell culture:

- Refresh growth medium the next morning after establishing a culture from cryopreserved cells to remove unattached cells. Change the medium every other day thereafter.
- Subculture the cells when the culture reaches around 90% confluency.

Subculture of the primary human liver endothelial and sinusoidal cells:

Note: Before the split, prepare a coated plate with fibronectin at 4ug/cm^2.

1. Before starting the subculture protocol, MEG has to be warmed at 37°C in a water bath and the TrypLE Express solution and DPBS at room temperature.
2. Aspirate the medium, then rinse the monolayer of cells with DPBS and remove the solution by aspiration.
3. Add TrypLE Express solution into the cell culture dish, rock it gently to ensure the solution covers all the cells and incubate in a 37˚C incubator until the cells detach. To monitor the detachment progress, use an inverted microscope until cells become rounded.

   Note: To improve the detachment, release the rounded cells from the culture surface by hitting the side of the flask against your palm until most of the cells are detached.

4. Transfer the cell suspension from the cell culture dish to a sterile centrifuge tube and wash the plate with MEG to recover the maximum number of cells detached. Then, centrifuge the tube at 430g for 7 minutes.
5. After centrifugation, aspirate the supernatant, resuspend in 1-2 ml of fresh medium and count cells for seeding (see section: determination of viability and number of cells).

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6. Seed the cells at a density of 10.000-15.000 cells/cm^2 on precoated fibronectin plates.

Cryopreservation of the primary human liver endothelial and sinusoidal cells:

1. Before starting the cryopreservation protocol, MEG has to be warmed at 37 °C in a water bath and the TrypLE Express solution, and DPBS at room temperature.

2. Aspirate the medium, then rinse the monolayer of cells with DPBS and remove the solution by aspiration.

3. Add TrypLE Express solution into the cell culture dish, rock it gently to ensure the solution covers all the cells and incubate in a 37˚C incubator until the cells detach. To monitor the detachment progress, use an inverted microscope until cells become rounded.

   Note: To improve the detachment, release the rounded cells from the culture surface by hitting the side of the flask against your palm until most of the cells are detached.

4. Transfer the cell suspension from the cell culture dish to a sterile centrifuge tube and wash the plate with MEG to recover the maximum number of cells detached. Then, centrifuge the tube at 430g for 7 minutes.

5. After centrifugation, aspirate the supernatant, resuspend in 1-2 ml of fresh medium and count cells for cryopreservation (see section: determination of viability and number of cells).

6. After counting the cells, centrifuge the cells and resuspend them in MLC to the required cell density.

   Note: Work quickly to maintain the cellular viability.

7. Put the cell suspension from step 6 in cryovials

8. Transfer them into a cryocooler (at room temperature) and put into a -80°C freezer.

   Note: The cryocooler will guarantee that the temperature declines steadily by 1°C/minute.

9. After approximately 24 h, remove the cryovials from the cryocooler and transfer into liquid nitrogen for long-term storage.

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