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3D-SPHEROID CULTURE
OF HUMAN PRIMARY HEPATOCYTES

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PROCEDURE

The aim of the present method is to describe the process of 3D-spheroid culture of human cryopreserved hepatocytes.

Please read through this entire protocol before attempting this procedure.

Recommended reagents and consumables

- Thawing and culture media (references)
  - **MHT**: Hepatocyte Thawing Medium
  - **3D-MHP**: 3D-Hepatocyte Plating Medium
  - **3D-MHM**: 3D-Hepatocyte Maintenance Medium
- 96-Well ULA round bottom cell culture plates (e.g. Greiner Cat. No. 650 979, Corning Cat. No. 7007)

1. Arrival of the cryopreserved cells in your facilities

Place the cryogenic hepatocyte vials immediately into the gas phase of liquid nitrogen tank.

2. Thawing and culture protocol for cryopreserved primary human hepatocytes

Hepatocyte Thawing Medium

The Hepatocyte Thawing Medium (MHT) is the combination of reagent A + B and it must be prepared the same day it is going to be used.
The following table specifies the volumes required to prepare 50 mL of thawing medium; bearing in mind that 50 mL of this medium will be used for a maximum of 3 cryovials.

Reagent	Quantity/Volume
A	35 mL
B	15 mL

Table 1. Thawing medium reagents volume.

Once the MHT has been prepared, it should be kept at 4°C until use, and it must be used the same day it has been prepared.

Thawing of primary human hepatocytes

1. Before starting the thawing process, MHT and 3D-MHP have to be warmed to 37 °C in a water bath.
2. Remove the cryopreserved hepatocytes from the liquid nitrogen and place it immediately into the 37 °C warm water bath until the cell suspension is thawed (approx. 1-2 min).
3. Wipe the cryovial with 70% ethanol for disinfection.
4. Pour or pipette the cell suspension contained in the cryovial into the 50 mL tube with preheated MHT (at most 3 vials per 50 mL).
5. Add 1 mL of MHT to each cryovial, in order to recover the cells that have remained inside each cryovial. Then, combine it with the cells in the 50 mL tube to ensure that all hepatocytes have been transferred.
6. Invert the tube slowly 2 or 3 times to mix the hepatocytes with the MHT (do not vortex).
7. Centrifuge at 100g for 8 min at RT.
8. Remove the supernatant and re-suspend the cell pellet with 3D-MHP by gently agitating the bottom of the tube. Do not vortex or shake the cells. Take care not to disrupt the pellet during the aspiration.

Determination the cell viability and yield

To determine cell viability and yield from the hepatocytes suspension in step 8, please follow these instructions:

- Carefully homogenize the cell suspension (do not vortex).
- Take 100 µL of the cell suspension and mix it in a 1.5 mL tube with 100 µL of Trypan Blue solution and 800 µL of PBS (1/10 dilution), and keep the rest of the cell suspension on ice in the laminar flow hood until seeding. Dilution can be modified considering the cell pellet.
- Gently homogenize the solution by inverting the tube with the hepatocytes and the Trypan Blue solution (do not vortex).
- Add an aliquot of 20 µL with the Trypan Blue/cell mixture into a cell counting chamber.
- Proceed to cell observation and counting under the microscope. Dead cells will appear in blue. In order to determine cell viability and yield, count the living and dead cells and use these formulas:

Viability (%) = (Live cell count/Total 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*

Seeding of 3D-Spheroid from primary human hepatocyte

ɹmber of cells

Maintenance of culture of Hepatocyte spheroids

1. Adjust cell suspension for plating of 2500 – 5000 cells/well in 100 µL/well 3D-MHP

2. Use 96-Well ULA round-bottom cell culture plates to seed the hepatocytes

3. Once the cells have been seeded, spin the plate at 500g for 2 min at RT to bring the plated cells to the center

4. Let the cells rest and form the spontaneous self-aggregation of the spheroids at 37°C and 5 % CO_2 (Figure 1).

5. Perform a daily light-microscopic control. Handle the plate very carefully to avoid any disturbance of the spheroid formation.

Note 1: To avoid medium evaporation, add 30 µL medium to the adjacent wells without cells

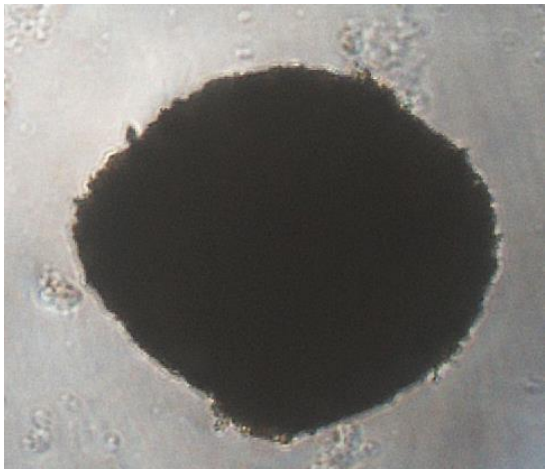

Figure 1. Hepatic spheroids spontaneous self-aggregation 5 days after seeding (image taken at 4x).

1. 5 days the seeding, 50% of the 3D-MHP will be replaced for 3D-MHM. Spin the plates again at 500g, RT, for 1 min.

2. From the sixth day after the seeding, change whole by slowly aspirating the old medium with a pipe and add 100 µL of fresh 3D-MHM.

   Note 2: the spheroid will most certainly float around the well, while pipetting. Try not to aspirate it. If the spheroid is aspirated, make sure to release it slowly into the well again before you continue. A dark background makes it easier to see the spheroids.

3. Once the cells formed one well-shaped spheroid per well, individual test-assays may be performed.

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