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PRIMARY ANIMAL
HEPATOCYTES CULTURE

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PROCEDURE

The aim of the present method is to describe the process of culturing animal cryopreserved hepatocytes.

Please read through this entire protocol before attempting this procedure.

Required and recommended reagents and consumables

- Thawing and culture media (references)
  - **MHT**: Hepatocyte Thawing Medium
  - **MHP**: Hepatocyte Plating Medium
  - **MHM**: Hepatocyte Maintenance Medium
- For the use of cryopreserved hepatocytes in suspension only MHT and MHP are required.
- Collagen I coated cell culture plates.
- If plating hepatocytes with an overlay, refer to the specification section for Matrigel coating which will provide the protocol and technical tips.

1. Arrival of the cryopreserved cells at your facilities

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

2. Thawing and culture protocol for cryopreserved primary animal hepatocytes

Hepatocyte Thawing Medium

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 animal hepatocytes

1. Before starting the thawing process, MHT and MHP must be warmed at 37°C.

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 partially 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, 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 RT the tubes with hepatocytes according to the information that appears in the table 2.

8. Remove the supernatant and re-suspend the cell pellet in 3-5 ml 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 and proceed to determine viability.

Category	Species	Centrifugation details
Mammals	Cynomolgus	100 x g for 10 min
	Beagle	
	Minipig	
	Landrace Pig	
	Horse	
	Rabbit	
	Sheep	200 x g for 10 min
	Mouse	50 x g for 10 min
	Rat	100 x g for 10 min
Birds	Chicken	200 x g for 10 min
	Turkey	100 x g for 10 min
	Duck	
Fishes	Rainbow Trout	100 x g for 10 min
	Atlantic Salmon	
	Common Carp	

Table 2. Centrifugation details for different animal species

Determining the cell viability and yield

To determine cell viability and yield from the hepatocyte's 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.
- 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*

- After determining cell viability and the total number of cells, proceed with cell seeding as follow:

Suspension Animal Hepatocytes:

- Adjust cell suspension to the desired density.
- Seed the cells in the desired well plate or microcentrifuge tube with MHP medium
- Incubate the cells at 37°C/5% CO_2
- **NOTE**: The use of thermoblock with shaker at 1000 rpm is recommended depending on the type of experiment to perform with suspension hepatocytes.

Format	Human	Volume/well
6 wells	0.80 x 10^6 cells/ml	2 ml
12 wells	0.75 x 10^6 cells/ml	1 ml
24 wells	0.70 x 10^6 cells/ml	500 µl
48 wells	0.50 x 10^6 cells/ml	200 µl
96 wells	0.70 x 10^6 cells /ml	100 µl

Table 3. Number of cells per well.

Plateable Animal Hepatocytes:

- After counting the cells, seed them in the desired collagen I coated cell culture plates with MHP (See Table 3). **The recommended seeding density for each lot is stated on the accompanying data sheet**.
- Adjust cell suspension to the desired density.
- Once the plates have been seeded shake vigorously in T form, except the 96-well plates that should NOT be shaken.
- Insert the cells in the incubator at 37°C/5% CO_2.
- During the first hour after the seeding, move the plate in T form every 15 minutes.
- Let the cells attach for at least 6-7 hours at 37°C/5 % CO_2. Note: Do not let the cells attach overnight.

Tips to seed the correct cell density:

In order to seed a well, we recommend the distribution of the cells evenly throughout by shaking the plate manually and check it under a microscope.

- **Low Density**: seed more cells in a well or proceed to prepare a more concentrated cell suspension by centrifuging the cells for 6 minutes at 100 g and resuspending the pellet in a smaller volume.
- **Correct density**: sow the rest of the plate following the data sheet of each lot.
- **High density**: dilute the cell suspension with medium plating and repeat the seed density checking process.

NOTE: For 96-well plates, initially dose 60 µL of pre-heated plating media and then add the cells to the well; Add 65 µL (0.7 x 106 cells/mL) and check it under a microscope, if the cell density is not suitable, fit it in another new well.

Culture of plateable primary animal hepatocytes

9. Once the plateable hepatocytes are adhered to the surface of the culture plate:

   a. If the overlay with Matrigel coating is not required for your culture, warm Hepatocyte Maintenance Medium (MHM) at 37°C and add to the cells according to the volume detailed in Table 2.

   b. If the overlay with Matrigel is required see section "Overlay matrix: Matrigel coating"

10. Change the MHM daily. Change the medium quickly and do not let the cells dry.

Overlay matrix: Matrigel coating

The solutions used should be kept at or below 4 °C.

1. Before coating with Matrigel, check that the hepatocytes are completely adhered to the surface of the well and that they are arranged forming a monolayer.

2. Depending on the format and the total number of plates to be coated with overlay matrix, Cytes Biotechnologies recommends making 2-5 % extra volume in case it is needed.

   Matrigel Volume = **(Required MHM Volume x 0.25 mg/ml) / Matrigel concentration**

3. Add and mix by pipetting the calculated Matrigel volume to the MHM required volume. As the Matrigel is very dense the pipetting must be slow.

4. Use the vacuum pump to aspirate the medium of all the plates.

5. Add the Matrigel solution required volume to each well (See volumes according to plate format in Table 3).

6. Reinsert the plates into the incubator at 37°C/5% CO_2.