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Protocol for Culture of Cryopreserved Human Hepatocytes

Cryopreserved adult and neonatal hepatocytes require special handling to ensure optimal viability following the freeze/thaw process. Please follow these methods closely to ensure the best performance of human hepatocytes in culture.

I. Required Equipment

1. All procedures should be conducted inside a biological safety cabinet such as a NuAire Class II Type A2.
2. Incubator should be controlled for humidity, temperature and CO_2 concentration such as a NuAire 4950 with the following settings
    a. 6% CO_2
    b. 95% humidity
    c. 37°C

    NOTE: It is best to fyrite incubators regularly to ensure appropriate gas concentration is maintained.

II. Required Supplies

1. Fresh or cryopreserved Human hepatocytes
2. Cryopreserved Hepatocyte Recovery Media (Cat.# CRM050)
3. Hepatocyte Plating Media (Cat.# HPM250)
4. Hepatocyte Culture Medium (Cat.# HCM50)
5. Collagen I coated plates (Sigma Cat.# C3867)
6. Liver biomatrix coated plates (Cat.# 12001-006. 12001-024, 12001-048, 12001-096)

III. Safety Working with Human Cells

1. Assume that all cells isolated from Human tissue are potentially hazardous even though they test negative for Human pathogens
2. Always wear personal protective equipment
    a. Wear appropriate lab coat, gloves and safety glasses
3. All operations should be conducted using aseptic technique within a biosafety cabinet

IV. Receiving Cryopreserved Human Hepatocytes and Human Stromal Cells

1. The cryopreserved hepatocytes are sensitive to temperature fluctuation, so they are stored in the vapor phase of liquid nitrogen and shipped in vapor phase of a liquid nitrogen cryo-shipper.
2. Cryopreserved stromal cells are less sensitive to temperature fluctuations so when they are shipped alone, they will arrive on dry ice, if shipped together with hepatocytes they will arrive in the cryo-shipper.
3. Upon receipt of the cells, immediately transfer the vials into your liquid nitrogen freezer.
4. Return the cryo-shipper the next day. Return documents are included in the shipment.

V. Making Hepatocyte Plating Medium

1. Hepatocyte Plating Medium (Cat.# HPM250 includes the following components:
    a. Hepatocyte Plating basal medium
    b. Hepatocyte Plating Frozen Supplement

2. Prepare Hepatocyte Plating Medium according to the instructions that accompany the media and supplement pack.

VI. Making Hepatocyte Culture Medium (HCM500)

1. Hepatocyte Culture Medium (Cat.# HCM500) includes the following components:
    a. Hepatocyte Culture basal medium 500 ml
    b. Hepatocyte Culture Frozen Supplement

2. Prepare Hepatocyte Culture Medium according to the instructions that accompany the media and supplement pack.

VII. Coating Plates with Collagen I

1. Make a solution of 50 µg/ml Collagen I by diluting the collagen in sterile cell culture grade water then filter through .2 µm filter unit.
2. Add a volume of 50 µg/ml Collagen I solution to each well and let set in hood for 2 hours. (use Table 1 for coating volumes)
3. Remove collagen solution and wash once with sterile water then add cells or let dry and store in 4°C for up to 1 year.

Table 1. Volumes for Coating Tissue Culture Dishes and Plates

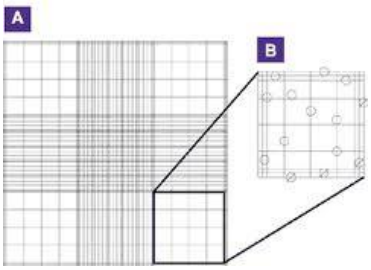
Dish/Flask Size	Growth Area (cm^2)	Coating Volume
6-Well	9.6	1.5 ml
12-Well	3.8	300 µl
24-Well	2.0	200 µl
48-Well	1.1	100 µl
96-Well	.32	50 µl
384-Well	.136	30 µl

VIII. Thawing Cryopreserved Human Hepatocytes

1. The night before thawing the hepatocytes remove the Hepatocyte Cryo Recovery Media (CRM050 from the freezer and place in the refrigerator to thaw overnight.
2. Determine the number of hepatocytes you will need for your experiment using plating densities per well from Table 2 then thaw appropriate number of vials.
3. If plating on liver biomatrix plates, at least 2 hours prior to thawing hepatocytes prepare the biomaxtrix plates according to the instructions.
4. If plating on Collagen I coated plates already made, no preparation prior to thawing is required.
5. Warm Hepatocyte Plating Media (HPM) to room temperature prior to thawing the cells, disinfect bottle with 70% ethanol and transfer into the biosafety cabinet.
6. Immediately prior to thawing the cells remove Hepatocyte Cryo Recovery Media (CRM) from the refrigerator, disinfect tube with 70% ethanol and transfer into the biosafety cabinet.
7. To thaw hepatocytes, remove the vial of cells from the LN_2 freezer and take it immediately to the

water-bath. If the freezer is not in close proximity to the water-bath, transport the vial of cells in dry ice.

8. Holding onto the cap, submerge the vial into the water continuously moving the vial in the water to ensure the water surrounding the vial remains warm.
9. Every 5-10 seconds remove the vial from the water and gently turn upside down to see if the frozen cell suspension has thawed. When the frozen cell suspension becomes a slurry take to the biosafety cabinet.
10. Douse the vial in 70% ethanol dry and place inside the biosafety cabinet.
11. Open the vial and using a 1-5 ml pipette gently remove the cell suspension from the vial and transfer into the 50 ml tube containing the CRM thawing media. You may transfer 2 vials of hepatocytes into one tube of CRM. Make sure to submerge the tip of the pipette below the surface of the media and gently express the cell suspension into the media.
12. Wash the vial with 1ml CRM media. Let the thawed hepatocytes rest in the hood for 15 minutes then pellet the cells by centrifugation at 100 x g for 5 minutes.
13. Remove the supernatant taking care not to disturb the cell pellet.
14. Add 5 ml HPM plating media and resuspend the pellet by gentle pipetting. If you have multiple tubes of hepatocytes you can pool them at this time so you take one count of the total hepatocyte population.
15. Take an aliquot for cell counting to determine cell count and percent viability
    a. Take 20ul cell suspension and add it to 20ul Trypan Blue. This provides a dilution factor of 2.
    b. Gently pipette up and down to produce a homogeneous cell suspension
    c. Take 10ul and add to the counting chamber of a hemacytometer
    d. Count the live cells and then count the blue (dead) cells within at least 2 corner grids



Cell count / 2 X 10,000 X 2 X Volume = Total cells

Cell count is divided by 2 for the 2 grids counted

10,000 is a constant for the surface area of the counting chamber

2 is the dilution factor

Volume is the volume of the cell suspension being counted

16. Calculate the total cells for each the live cells and the dead cells
17. To determine percent live cells
    a. Total live cell count + total dead cell count = total cells
    b. Total Live cell count ÷ total cells x 100 = percent live cells
18. Let the hepatocytes set in the biosafety cabinet while you thaw the stromal cells.

Table 2. Plating densities for Human hepatocytes.

Dish/Flask Size	Growth Area (cm^2)	Media Volume (ml) per well	Hepatocytes per well
6-Well	9.6	2	1.44×10^6
12-Well	3.8	1	5.70×10^5
24-Well	2.0	0.5	3.00×10^5
48-Well	1.1	0.2	1.65×10^5
96-Well	.32	0.1	4.80×10^4
384-Well	.136	0.05	2.04×10^4

IX. Plating Human Hepatocytes

1. Based on the number of wells you need to seed on each plate and the number of plates you will need for the experiment use the cells/well values in Table 2 to calculate total hepatocytes and stromal cells required for the experiment.

(Hepatocytes/Well from Table 2) x (# Wells) x (# Plates) = Total Hepatocytes

2. Resuspend hepatocytes in an appropriate volume of HPM plating media required for seeding the cells into the wells:

(Media Volume/Well from Table 2) x (# wells) x (# plates) = Total HPM Volume

3. Seed the wells and transfer the plates into the incubator. Next day do a complete media change.

XI. Feeding Human Hepatocytes

1. Hepatocytes should be fed every day for optimal maintenance of metabolic activity.
2. Make Hepatocyte Culture Media (HCM) according to product instructions.
3. Warm HCM media to at least room temperature. If warming in water-bath, don't leave in water-bath longer than 5 minutes, as proteins and growth factors easily degrade when left in water bath for extended periods of time.
4. Remove the plates from the incubator and transfer to the biosafety cabinet. Remove the old media from the cells and replace with fresh HCM media by slowly adding the media against the side of the well then return the plate to the incubator. Media volume per well is listed in Table 2. Do not add the media directly to the cell monolayer.
5. Repeat feeding daily.

