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Recovery and Culture of Cryopreserved Hepatocytes

Cryopreserved hepatocytes require special handling for optimal performance. They are stored and shipped in liquid nitrogen. Upon receiving cryopreserved hepatocytes immediately transfer into a liquid nitrogen freezer until ready for use. Our hepatocyte media has been formulated for optimal recovery, plating and maintenance of cryopreserved hepatocytes. Follow the methods below for optimal recovery and long term culture of cryopreserved hepatocytes.

Materials Required

Media – Follow instructions that accompany the media

- Cryo Recovery Medium (Cat.# CRM050) – Ready to use when thawed
- Hepatocyte Plating Media (Cat.# HPM250)
- Hepatocyte Culture Medium (Cat.# HCM500)

Collagen I or Liver Biomatrix Coated Plates

- 50µg/ml Collagen I (Sigma C3867) in sterile cell culture water
- Multi-well plates
- Liver Biomatrix Coated Plates (See PSB Catalog for ordering plates/suspension)

Other Material

- 50ml conical tubes
- Cryopreserved Hepatocytes (QPS)

Required Equipment

- All procedures should be conducted inside a biological safety cabinet such as a NuAire Class II Type A2.
- Human hepatocytes can be maintained with an antibiotic if required in order to prevent bacterial contamination (not included in the media).
- Incubator such as the NuAire 4950 should be set with the following settings
    - 6% CO_2
    - 95% humidity
    - 37°C

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

I. Coating Plates with Collagen I

1. Make a solution of 50µg/ml Collagen I by diluting the collagen in sterile cell culture grade water then filter through .2µm filter unit.
2. Add a volume of 50µg/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° for up to 1 year.

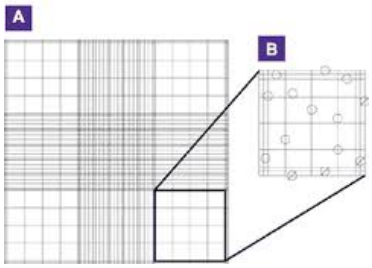
Table 1. Volumes for Coating Tissue Culture Dishes and Plates

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

II. Thawing Cryopreserved Human Hepatocytes

1. The day prior to thawing the hepatocytes, place the frozen Cryo Hepatocyte Recovery Medium (CRM-050) in 4°C refrigerator to thaw overnight.
2. Determine the number of hepatocytes you will need for your experiment using plating densities per well from Table 2.
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 Medium (CRM) from the refrigerator, disinfect tube with 70% ethanol and transfer into the biosafety cabinet.
7. Transfer the CRM from the bottle into a 50ml conical tube.
8. To thaw hepoatocytes, 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.
9. 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.
10. 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.
11. Douse the vial in 70% ethanol dry and place inside the biosafety cabinet.
12. 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 recovery 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.
13. 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.
14. Remove the supernatant taking care not to disturb the cell pellet. 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 prepare for plating.

III. Plating Human Hepatocytes/Stromal Cell Co-Culture

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 required for the experiment.

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

- Add HPM to the hepatocytes in an appropriate volume required for seeding the cells into the wells:

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

2. Seed the wells and transfer the plates into the incubator. First thing the next day you will do a complete media change as in step IV below.

Table 2. Plating densities for human hepatocytes

Dish/Flask Size	Growth Area (cm^2)	Plating Volume (ml)	Hepatocytes per well
6-Well	9.6	2	1.50×10^6
12-Well	3.8	1	7.50×10^5
24-Well	2.0	0.5	3.75×10^5
48-Well	1.1	0.2	1.88×10^5
96-Well	.32	0.1	4.88×10^4
384-Well	.136	0.05	2.55×10^4

IV. 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 the same as the plating volume in Table 2. Do not add the media directly to the cell monolayer.
5. Repeat feeding daily

