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Cryopreserved Neonatal Hepatocytes Methods

Cryopreserved 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 the hepatocytes.

I. Required Equipment

1. All procedures should be conducted inside a biological safety cabinet such as a NuAire Class II Type A2.
2. Hepatocytes can be maintained with an antibiotic if required in order to prevent bacterial contamination. Our cells have been tested with gentamycin (30μg/ml).
3. 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. Hepatocyte Culture Medium (PSB Cat.# HCM500)
2. Hepatocyte Thawing Medium (PSB Cat.# CRM050)
3. Hepatocyte Plating Medium (PSB Cat.# HPM250)
4. Liver Biomatrix Coated Plates or Liver Biomatrix Suspension

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 Hepatocytes

1. The cryopreserved hepatocytes are sensitive to temperature fluctuations so they are stored in the vapor phase of liquid nitrogen and shipped in vapor phase of a liquid nitrogen cryo-shipper.
2. Upon receipt of the shipper, immediately transfer the vials into your liquid nitrogen freezer.
3. Return the cryo-shipper the next day. Return documents are included in the shipment.

V. Making Hepatocyte Maintenance Medium

1. Hepatocyte Maintenance Medium (Cat.# HCM500) includes the following components:
    a. Hepatocyte Maintenance basal medium

b. Hepatocyte Maintenance Frozen Supplement

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

VI. Thawing Cryopreserved Neonatal Hepatocytes

1 . At least 2 hours prior to thawing hepatocytes prepare the Liver Biomaxtrix plates according to the instructions. If using Liver Biomatrix Suspension, plates will need to be made the day before thawing hepatocytes.
2 . Warm Hepatocyte Maintenance Media and Hepatocyte Thawing Media to room temperature.
3 . To thaw the cells, 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.
1. 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.
2. 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 cell suspension becomes a slurry take to the safety cabinet
3. Douse the vial in alcohol dry and place inside the safety cabinet.
4. Open the vial and using a 1-2ml pipette gently remove the cell suspension from the vial.
5. Transfer the cell suspension into the Thawing Media. Make sure to submerge the tip of the pipette below the surface of the media and gently express the cell suspension into the media.
6. Wash the vial with 1ml Thawing Media then pellet the cells by centrifugation at 100g for 5 minutes.
7. Remove the supernatant taking care not to disturb the cell pellet.
8. Add 10ml Hepatocyte Maintenance Media and resuspend the pellet by gentle pipetting
9. 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 the center grid

Cell count X 10,000 X 2 X Volume = Total cell count

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

14. Calculate the total cell count for each the live cells and the dead cells
15. 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
16. Determine cell density for plating neonatal hepatocytes using Table 1.

Table 1. Plating densities for neonatal hepatocytes plated at confluence (1.88×10^5 cells/cm^2).

Dish/Flask Size	Growth Area (cm^2)	Plating Volume (ml)	Maximum Volume (ml)	Hepatocytes per well
6-Well	9.6	2	3	1.50×10^6
24-Well	2.0	0.5	1.5	3.75×10^5
96-Well	.32	0.1	.2	4.88×10^4

17. Plate neonatal hepatocytes onto Liver Biomatrix coated plates.
18. First thing the next morning, remove the media from the cells and replace with fresh Hepatocyte Maintenance Media.

VII. Feeding Neonatal Hepatocytes

1. Hepatocytes should be fed every day for optimal maintenance of metabolic activity.
2. Warm Hepatocyte Maintenance 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.
3. Remove the plate from the incubator and transfer to the safety cabinet. Remove the old media from the cells and replace with fresh Hepatocyte Maintenance Media by slowly adding the media against the side of the dish then return the plate to the incubator. Do not add the media directly to the cell monolayer.

