Protocol for Thawing and Use of Plateable and Suspension Cryopreserved Hepatocytes

Introduction

This protocol covers the thawing and prep of cryopreserved hepatocytes for their subsequent use in applications such as metabolic stability (intrinsic clearance), metabolite ID/profiling, enzyme induction, hepatotoxicity, transporter uptake and efflux, environmental bioaccumulation and liver disease research. The initial part of this protocol is suitable for suspension lots; follow the entire protocol for plating and overlay of plateable hepatocytes.

(For a complete list of our cryopreserved hepatocytes, visit www.invitrogen.com/hepatocytes)

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Recommended reagents	Size	Cat. No.
Human suspension		
→ Thawing Medium		
• Hepatocyte Thawing Medium (HTM)	50 mL	CM7500
→ Incubation Medium		
• Williams' Medium E (1x, no phenol red)	500 mL	A1217601
• Hepatocyte Maintenance Supplement Pack (Serum-free)	1 pack	CM4000
Human plateable		
→ Thawing Medium		
• Hepatocyte Thawing Medium (HTM)	50 mL	CM7500
→ Plating Medium		
• Williams' Medium E (1x, no phenol red)	500 mL	A1217601
• Hepatocyte Plating Supplement Pack (Serum-containing)	1 pack	CM3000
→ Incubation Medium		
• Williams' Medium E (1x, no phenol red)	500 mL	A1217601
• Hepatocyte Maintenance Supplement Pack (Serum-free)	1 pack	CM4000
• Collagen I, Coated Plates	1 plate	CM1024
• Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix	1 mL	A1413201
Animal suspension		
→ Thawing Medium		
• Hepatocyte Thawing Medium (HTM)	50 mL	CM7500
→ Incubation Medium		
• Williams' Medium E (1x, no phenol red)	500 mL	A1217601
• Hepatocyte Maintenance Supplement Pack (Serum-free)	1 pack	CM4000
Animal plateable		
→ Thawing Medium		
• Hepatocyte Thawing Medium (HTM)	50 mL	CM7500
→ Plating Medium		
• Williams' Medium E (1x, no phenol red)	500 mL	A1217601
• Hepatocyte Plating Supplement Pack (Serum-containing)	1 pack	CM3000
→ Incubation Medium		
• Williams' Medium E (1x, no phenol red)	500 mL	A1217601
• Hepatocyte Maintenance Supplement Pack (Serum-free)	1 pack	CM4000
• Collagen I, Coated Plates	1 plate	CM1024
• Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix	1 mL	A1413201

(See www.invitrogen.com/hepatocytes for alternative sizes and options)

Advance preparation

- → If plating hepatocytes with an overlay, refer t o the specification sheet for Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (cat. no. A1413201), which will provide the lot's concentration and technical tips. Geltrex™ Matrix should be thawed on ice for 2-3 hours prior to application, or overnight at 4ºC, and kept ice-cold to prevent gelling.
- → Read the directions supplied with the Hepatocyte Maintenance and Plating Supplement Packs (CM4000 and CM3000) and prepare Maintenance and Plating Media using Williams' Medium E.
- → Review this protocol to ensure you have all the necessary reagents and equipment prior to starting the procedure. Once thawed, cryopreserved hepatocytes must be used immediately and will not retain metabolic activities if re-frozen.
- → Not all cryopreserved hepatocytes are suitable for plating. If using this protocol for plating hepatocytes, confirm that the lot is plateable. (**Note**: Use universal safety precautions and appropriate biosafety cabinet when handing primary hepatocytes.)

Protocol

Thaw, spin, resuspend

1. Warm these media to 37°C in a water bath:
   - → HTM for human or animal hepatocytes
   - → Plating Medium (if plating hepatocytes; this is Williams' Medium E supplemented with Hepatocyte Plating Supplement Pack, Serum-Containing)
   - → Incubation Medium (for suspension only this is Williams' Medium E supplemented with Hepatocyte Maintenance Supplement Pack, Serum-free)
2. Thaw cryopreserved hepatocytes in 37°C water bath for <2 min.
3. Wipe the vial with 70% alcohol in hood; pour or use wide-bore pipette tip to transfer hepatocytes into HTM.
4. Centrifuge at room temperature:
   - → Human hepatocytes, 100 x *g* for 10 min.
   - → Animal hepatocytes, 86 x *g* for 6 min.
5. Pour supernatant off into waste bottle and invert completely. Do not shake. Add ~1 ml of the following per 1 x 10^6 total cells:
   - → Plating Medium, if plating the hepatocytes
   - → Pre-warmed Incubation Medium, if using cells in suspension

Count, plate, and incubate

6. Determine cell viability and yield. For help, see:
   - → Cell Counting Calculation Worksheet

   (**Note**: Hepatocytes are very fragile and often automated cell counting instruments will give false viabilities and yields. We suggest manual counting for better accuracy, as the correct plating density is critical for good results.)
7. ***If using the hepatocytes in suspension, add additional medium to bring cells to desired concentration (i.e. 1x10^6 cells/mL) — do not proceed with the subsequent plating steps.***

(Protocol continued on pg. 4)

8. Dilute to correct seeding density with Plating Medium. (Table 1, Table 2)
9. Pipet cells into multi-well plate, with a serological pipet or wide-bore pipet tips. Resuspend the hepatocyte stock every few wells to ensure a homogeneous mixture.
10. Place plate in incubator, and with hand on top of lid disperse cells with north/south and east/west motions.
11. Incubate plate at 37°C for 4-6 hr.
    → Do not move/disturb plate during this time, as the cells are forming a monolayer—cells will pool to middle of plate if agitated.

    (**Note**: If not overlaying, prior to feeding plates 4-6 hours later, pre-warm Incubation Medium (this is Williams Medium E supplemented with GIBCO® Hepatocyte Maintenance Supplement Pack (Serum-free)).

    (**Note**: If you are using an overlay, the Inubation Medium needs to be kept ice cold. See the overlay section for more information.)
12. After incubation, shake plate on hood surface to loosen debris and aspirate medium.
13. If using an overlay, proceed to the next step. If not using an overlay, replace medium with pre-warmed Incubation Medium, or alternative medium, depending on your application. Do not let the hepatocytes dry out—replace medium quickly.

Overlay

(**Important Note**: Geltrex™ Matrix and the Incubation Medium used for its dilution must be kept ice cold to prevent premature gelling. Keep Geltrex™ Matrix and Incubation Medium on ice; preferably use cold pipettes when mixing.)

14. Calculate the amount of Incubation Medium needed to feed the plated hepatocytes and place this volume on ice.
    → Generally, this is 12 mL per plate; consider adding 1-2 mL for a slight excess of solution.
15. Find the protein concentration of Geltrex™ Matrix on its specification sheet—each lot is slightly different.
16. Multiply the amount of Incubation Medium by our recommended final Geltrex™ Matrix concentration of 0.35 mg/mL, and divide by the protein concentration of Geltrex to get the amount of Geltrex™ Matrix that needs to be added to the Incubation Medium:
    → (mL Incubation Medium x 0.35 mg/mL) / Geltrex protein conc. = mL of Geltrex to add
17. Add Geltrex™ Matrix to the cold Incubation Medium on ice. Mix well by pipeting several times and invert media to ensure homogeneous solution.
18. Apply overlay to plated hepatocytes and incubate at least two hours or up to 24 hours prior to use.
    → The gel layer will settle out of the media over the top of the hepatocytes
19. Replace Incubation Medium daily.

(**Note**: For overlaying dog hepatocytes we recommend using Matrigel™ (BD) or ECM (Sigma))

Technical support

For questions related to this protocol, contact us at:

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Table 1—General Seeding Density Guide for Cryopreserved Hepatocytes. 12 mL media per plate. **Note**: each lot may require slight adjustments in seeding density to form optimal monolayer. Please contact Technical Support with any questions – 1-866-952-3559.

Species	6-well	12-well	24-well	48-well	96-well
Human	0.9-1.1 x 10^6 cells/mL	0.8-1.0 x 10^6 cells/mL	0.7-0.9 x 10^6 cells/mL	0.6-0.8 x 10^6 cells/mL	0.5-0.7 x 10^6 cells/mL
Rat	0.9-1.1 x 10^6 cells/mL	0.8-1.0 x 10^6 cells/mL	0.7-0.9 x 10^6 cells/mL	0.6-0.8 x 10^6 cells/mL	0.5-0.7 x 10^6 cells/mL
Dog	0.9-1.1 x 10^6 cells/mL	0.8-1.0 x 10^6 cells/mL	0.7-0.9 x 10^6 cells/mL	0.6-0.8 x 10^6 cells/mL	0.5-0.7 x 10^6 cells/mL
Monkey	1.1-1.3 x 10^6 cells/mL	1.0-1.2 x 10^6 cells/mL	0.9-1.1 x 10^6 cells/mL	0.8-1.0 x 10^6 cells/mL	0.7-0.9 x 10^6 cells/mL
Mouse	0.5-0.7 x 10^6 cells/mL	0.4-0.6 x 10^6 cells/mL	0.3-0.5 x 10^6 cells/mL	0.2-0.4 x 10^6 cells/mL	0.1-0.3 x 10^6 cells/mL

Table 2—Approximate Number of Cells Per Plate. 12 mL media per plate.

Species	6-well	12-well	24-well	48-well	96-well
Human	10.8-13.2 million cells	9.6-12 million cells	8.4-10.8 million cells	7.2-9.6 million cells	6-8.4 million cells
Rat	10.8-13.2 million cells	9.6-12 million cells	8.4-10.8 million cells	7.2-9.6 million cells	6-8.4 million cells
Dog	10.8-13.2 million cells	9.6-12 million cells	8.4-10.8 million cells	7.2-9.6 million cells	6-8.4 million cells
Monkey	13.2-15.6 million cells	12-14.4 million cells	10.8-13.2 million cells	9.6-12 million cells	8.4-10.8 million cells
Mouse	6-8.4 million cells	4.8-7.2 million cells	3.6-6 million cells	2.4-4.8 million cells	1.2-3.6 million cells