Thawing and Use of Plateable and Suspension Cryopreserved Hepatocytes

Hepatocyte Thaw Medium Method

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

IMPORTANT! Use universal safety precautions and appropriate biosafety cabinet when handing primary hepatocytes.

Introduction

This user bulletin covers the thawing and preparation 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. Methods are described for suspension lots and for plating and overlay of plateable hepatocytes.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Item	Source
Hepatocyte Thaw Medium	CM7500
William's E Medium, no phenol red	A1217601
Primary Hepatocyte Maintenance Supplements	CM4000
Primary Hepatocyte Thawing and Plating Supplements	CM3000
Collagen I, Coated Plate	A11428
Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	A1413201

Procedural guidelines

- If plating hepatocytes with an overlay, refer to the specification sheet for Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413201), which will provide the lot concentration and technical tips. Geltrex™ Matrix should be thawed on ice for 2–3 hours prior to application, or overnight at 4°C, and kept ice-cold to prevent gelling.
- Read the instructions for the Primary Hepatocyte Maintenance Supplements (Cat. No. CM4000) and Primary Hepatocyte Thawing and Plating Supplements (Cat. No. CM3000) and prepare Maintenance and Plating Media using William's E Medium.
- 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.

Reagents reference

Reagent	Amount
Human suspension	
Thawing Medium:	
Hepatocyte Thaw Medium	50 mL
Incubation Medium:	
William's E Medium, no phenol red	500 mL
Primary Hepatocyte Maintenance Supplements	1 pack
Human plateable	
Thawing Medium:	
Hepatocyte Thaw Medium	50 mL
Plating Medium:	
William's E Medium, no phenol red	500 mL
Primary Hepatocyte Thawing and Plating Supplements	1 pack
Incubation Medium:	
William's E Medium, no phenol red	500 mL
Primary Hepatocyte Maintenance Supplements	1 pack
Collagen I, Coated Plate	1 plate
Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	1 mL
Animal suspension	
Thawing Medium:	
Hepatocyte Thaw Medium	50 mL
Incubation Medium:	
William's E Medium, no phenol red	500 mL
Primary Hepatocyte Maintenance Supplements	1 pack
Animal plateable	
Thawing Medium:	
Hepatocyte Thaw Medium	50 mL
Plating Medium:	
William's E Medium, no phenol red	500 mL
Primary Hepatocyte Thawing and Plating Supplements	1 pack
Incubation Medium:	
William's E Medium, no phenol red	500 mL
Primary Hepatocyte Maintenance Supplements	1 pack
Collagen I, Coated Plate	1 plate
Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	1 mL

Thaw, spin, resuspend

1. Warm media to 37°C in a water bath:
   - Hepatocyte Thaw Medium for human or animal hepatocytes
   - Plating Medium (if plating hepatocytes; this is William's E Medium supplemented with Primary Hepatocyte Thawing and Plating Supplements)
   - Incubation Medium (for suspension only, this is William's E Medium supplemented with Primary Hepatocyte Maintenance Supplements)
2. Thaw cryopreserved hepatocytes in 37°C water bath for <2 minutes.
3. Wipe the vial with 70% alcohol in the hood; pour or use wide-bore pipette tip to transfer hepatocytes into Hepatocyte Thaw Medium.
4. Centrifuge at room temperature:
   - Human hepatocytes, 100 × *g* for 10 minutes.
   - Dog hepatocytes, 100 × *g* for 10 minutes.
   - Monkey hepatocytes, 110 × *g* for 10 minutes.
   - Rat hepatocytes, 100 × *g* for 10 minutes.
   - Mouse hepatocytes, 80 × *g* for 10 minutes.
5. Pour supernatant off into waste bottle and invert completely.

   Do not shake.
6. Add ~3 mL of the following media and gently resuspend the cells by gently rocking the tube front and back until no cell clump is observed. Avoid mixing hepatocytes by rapid pipetting which can cause cellular damage.
   - Plating Medium, if plating the hepatocytes
   - Pre-warmed Incubation Medium, if using cells in suspension

Count, plate, and incubate cells

1. Manually count live and dead cells using hemocytometer. Determine cell viability and yield.

   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.
2. If using the hepatocytes in suspension, add additional medium to bring cells to desired concentration (i.e. 1×10^6 cells/mL) — do not proceed with the subsequent plating steps.
3. Dilute to correct seeding density with Plating Medium. (Table 1, Table 2).
4. Pipet cells into multi-well plate, with a serological pipet or wide-bore pipet tips.

   Note: If 96-well plate is used to culture cells, pre-wet the plate by adding 60 µL of Plating Medium to each well, then add 65 µL of hepatocyte stock to each pre-wetted well for a total of 125 µL media per well.

   Resuspend the hepatocyte stock every few wells to ensure a homogeneous mixture.
5. Place plate in incubator, and with hand on top of lid disperse cells with north/south and east/west motions.
6. Incubate plate at 37°C for 4–6 hours.

   Do not move/disturb plate during this time to allow cells to settle and form 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 William's E Medium supplemented with Primary Hepatocyte Maintenance Supplements (Serum-free)).

   Note: If you are using an overlay, the Incubation Medium needs to be kept ice cold. See the overlay section for more information.
7. After incubation, shake plate on hood surface to loosen debris and aspirate medium.
8. 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! Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane 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.

1. 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.
2. Find the protein concentration of Geltrex™ matrix on its specification sheet—each lot is slightly different.
3. 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™ matrix to get the amount of Geltrex™ matrix that needs to be added to the Incubation Medium:
   - For Human or Monkey: (mL Incubation Medium × 0.35 mg/mL) / Geltrex™ protein conc. = mL of Geltrex™ matrix to add
   - For Rat or Mouse: (mL Incubation Medium × 0.6 mg/mL) / Geltrex™ protein conc. = mL of Geltrex™ matrix to add
   - For Dog: (mL Incubation Medium × 0.25 mg/mL) / Geltrex™ protein conc. = mL of Geltrex™ matrix to add

4. Add Geltrex™ matrix to the cold Incubation Medium on ice. Mix well by pipeting several times and invert media to ensure homogeneous solution.

5. 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.

6. Replace Incubation Medium daily.

Plating guidelines

Table 1 General seeding density guide for cyropreserved hepatocytes

12 mL media per plate. Each lot may require slight adjustments in seeding density to form optimal monolayer.

Species	6-well	12-well	24-well	48-well	96-well
Human	0.9–1.1 × 10^6 cells/mL	0.8–1.0 × 10^6 cells/mL	0.8–1.0 × 10^6 cells/mL	0.6–0.8 × 10^6 cells/mL	0.5–0.7 × 10^6 cells/mL
Rat	0.9–1.1 × 10^6 cells/mL	0.8–1.0 × 10^6 cells/mL	0.7–0.9 × 10^6 cells/mL	0.6–0.8 × 10^6 cells/mL	0.5–0.7 × 10^6 cells/mL
Dog	0.9–1.1 × 10^6 cells/mL	0.8–1.0 × 10^6 cells/mL	0.7–0.9 × 10^6 cells/mL	0.6–0.8 × 10^6 cells/mL	0.5–0.7 × 10^6 cells/mL
Monkey	1.1–1.3 × 10^6 cells/mL	1.0–1.2 × 10^6 cells/mL	0.9–1.1 × 10^6 cells/mL	0.8–1.0 × 10^6 cells/mL	0.7–0.9 × 10^6 cells/mL
Mouse	0.5–0.7 × 10^6 cells/mL	0.4–0.6 × 10^6 cells/mL	0.3–0.5 × 10^6 cells/mL	0.2–0.4 × 10^6 cells/mL	0.1–0.3 × 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 × 10^6 cells	9.6–12 × 10^6 cells	9.6–12.0 × 10^6 cells	7.2–9.6 × 10^6 cells	6–8.4 × 10^6 cells
Rat	10.8–13.2 × 10^6 cells	9.6–12 × 10^6 cells	8.4–10.8 × 10^6 cells	7.2–9.6 × 10^6 cells	6–8.4 × 10^6 cells
Dog	10.8–13.2 × 10^6 cells	9.6–12 × 10^6 cells	8.4–10.8 × 10^6 cells	7.2–9.6 × 10^6 cells	6–8.4 × 10^6 cells
Monkey	13.2–15.6 × 10^6 cells	12–14.4 × 10^6 cells	10.8–13.2 × 10^6 cells	9.6–12 × 10^6 cells	8.4–10.8 × 10^6 cells
Mouse	6–8.4 × 10^6 cells	4.8–7.2 × 10^6 cells	3.6–6 × 10^6 cells	2.4–4.8 × 10^6 cells	1.2–3.6 × 10^6 cells

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