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Product Instruction

Protocol for Thawing Cryopreserved Hepatocytes

The following procedure may be carried out in a biosafety containment hood to reduce the risk of contamination and also minimize contact with potentially biohazardous material.

Step-wise Procedure for Thawing Kits (K8000, K8100, K8500 & K8800)

This procedure describes the steps required for the isolation of hepatocytes using XenoTech's Hepatocyte Isolation Kits.

Kit Components:

Opti**Thaw** Hepatocyte Media
Opti**Count**: 50 µL Trypan Blue and 400 µL of 1xPBS

1) Warm the Opti**Thaw** media to 37 ± 1°C in water bath before use (typically takes ~15-20 minutes).

2) Remove the cryotube from the LN_2 storage unit and immediately place in a 37 ± 1°C water bath for ~80 seconds until the frozen cell pellet can move freely when the cryotube is inverted. *Do not over-thaw.*

Note: *For CryostaX™ pools, the contents of the vial can be immediately dispensed into the pre-warmed Opti**Thaw** Hepatocyte Media. Do not thaw CryostaX™ in a water bath. Once the frozen pellets are transferred to Opti**Thaw**, gently invert the tube until all of the pellets have melted.*

3) Transfer the frozen pellet from the cryotube into Opti**Thaw** Hepatocyte Media. Rinse each cryotube with 1.5 mL of Opti**Thaw**. Pour this rinse back into the Opti**Thaw** tube and gently invert until fully melted.

4) Centrifuge at 100 *x* g for 5 minutes at room temperature or 2-8°C.

- Aspirate and discard the supernatant fluid without disturbing the cell pellet.

5) Resuspend the cell pellet(s) with K8200 Opti**Plate** Hepatocyte Media (for plating hepatocytes) or K8400 Opti**Incubate** Hepatocyte Media (for suspension incubations), be careful not to over-dilute the cells based on final target cell concentration.

DO NOT VORTEX.

- Remove 50 µL of the homogenous cell suspension and dispense the 50 µL aliquot into the Opti**Count** tube.
- Mix gently. Cell viability can now be assessed by placing an aliquot from the counting tube on a hemacytometer and counting the dead (blue) cells and viable cell number.

6) Measure the volume of the cell suspension and *q.s.* to the desired concentration.

Cryopreserved Hepatocyte Sample Preparation Worksheet

This worksheet may be used to record information during the preparation of your hepatocyte sample. Prepare additional copies of this sheet as needed.

Hepatocyte Sample Identification

# Vials Thawed	
Sample ID (Species/Lot Number)	**1.5 mL**

Date of hepatocyte isolation: _____

Trypan Blue Cell Count Analysis

A trypan blue exclusion analysis should be performed (step 5 in the thawing protocol) following re-suspension of the initial cell pellet.

Cells Counted		% Viability [A/(A+B)] x 100	Dilution factor[1]	Hemacytometer factor[2]	Volume of sample[3]	Number of viable hepatocytes[4]	Final cell concentration[5]
Live	Dead						
A	B		C	D	E		
				10,000			
				10,000			

1. The dilution factor will equal 10 if a 50 µL aliquot of the cell suspension was dispensed into XenoTech's hepatocyte isolation Opti**Count** tube for subsequent counting. If an alternate means of dilution was used, a different dilution factor should be calculated.
2. The hemacytometer factor will typically equal 10,000. For more information consult your hemacytometer manufacturer.
3. Volume of the sample indicates the total volume of the cell suspension from which the counting aliquot was removed.
4. The number of viable hepatocytes may be calculated from the following equation:

$$\left(\frac{A}{\text{quadrants}}\right) \times C \times D \times E$$

where "quadrants" equals the number of quadrants counted on the hemacytometer.

5. The desired concentration should be determined based on the specific requirements of your experimental design.

Sample Dilution

Use the following table to calculate the final volume needed to reach the desired cell concentration

# of viable hepatocytes (determined above)	Desired cell concentration for use	Final volume	Volume of media to add to reach desired conc.
F	G	H	I

H = F/G I = H - Volume of sample

Tips for Working with Hepatocytes

- Thaw time is critical; over-thawing cryopreserved hepatocytes will result in low yield and viability. The solid, frozen pellet should be transferred directly into the Opti**Thaw** tube.
- For best results ensure level of water in the water bath is above the highest frozen point in the vial.
- When aspirating supernatant, keep tip of the aspirator at the highest level of media to ensure any cell debris is removed before reaching the viable cell pellet.
- XenoTech does not recommend pouring off supernatant, due to the high risk of losing the viable cell pellet during the pour process.
- Never vortex or vigorously resuspend the hepatocytes. A gentle rocking motion is recommended.
- We recommend performing two Trypan blue counts after centrifugation for verification of yield and viability.
- One hepatocyte isolation kit can be used to thaw up to 3 vials.

Suspension Incubations

Materials Needed:

- Flat-bottomed, uncoated incubation vessel
- Static or shaking incubator temperature: 37°C, Atmosphere: Rh (95%), CO_2 (5%), for long-term incubations a humidified incubator is recommended
- Opti**Incubate** Hepatocyte Media (K8400)

Typical final cell concentration per incubation	1 x 10^6 cells per mL
Flat-bottom incubation vessel	6-, 12-, 24-, 48-, 96- well
Typical incubation times	0, 30, 60, 90, 120, 240 min

Incubation media selection:

For long-term incubations (> 2 hours), Opti**Incubate** Hepatocyte Media (K8400) is recommended to maintain a higher viability throughout the incubation time.

If the test compound may be susceptible to protein binding, a buffer such as KHB is recommended because it does not contain any protein supplementation.

Krebs Henseleit Buffer (KHB)

pH 7.4 *no nutritional supplementation* (Sigma)

Protocol for Plating and Culturing Hepatocytes

Materials Needed:

- BioCoat™ Collagen I Cellware (BD Biosciences) or equivalent
- Cell Culture Incubator Temperature: 37°C Atmosphere: Rh (95%), CO_2 (5%)
- Opti**Culture** Media and Pen/Strep (K8300)*
- Opti**Plate** Hepatocyte Media (K8200)
- Opti**Matrix** Hepatocyte Overlay (K8600/K8650)

**K8300 comes with a supplemental vial of Pen/Strep. Add the entire contents of the vial to the bottle of media and update the expiration date to one month from the date of the addition.*

Procedure

1) Thaw hepatocytes as stated earlier in the protocol.

2) Dilute the hepatocyte suspension to the desired concentration with Opti**Plate** media. The table below shows a range of recommended seeding densities for each species. See the lot specific data sheet for a particular lot's recommended seeding density.

3) Add appropriate volume of cell suspension to each well. The table below provides recommended seeding volumes for the various species and plating formats.

Species	6-Well Format		12-Well Format		24-Well Format		48-Well Format		96-Well Format*	
	Recommended Seeding Density (million cells/mL)	Recommended Seeding/Feeding Volume Per Well	Recommended Seeding Density (million cells/mL)	Recommended Seeding/Feeding Volume Per Well	Recommended Seeding Density (million cells/mL)	Recommended Seeding/Feeding Volume Per Well	Recommended Seeding Density (million cells/mL)	Recommended Seeding/Feeding Volume Per Well	Recommended Seeding Density (million cells/mL)	Recommended Seeding/Feeding Volume Per Well
Human	1.0 - 1.6	1.7 mL	1.0 - 1.6	650 µL	1.0 - 1.6	330 µL	0.75	200 µL	0.75	75 µL
Rat	1.2 - 1.4	1.7 mL	1.2 - 1.4	650 µL	1.2 - 1.4	330 µL	1.2 - 1.4	150 µL	0.6 - 0.7	75 µL
Monkey	1.4 - 2.2	1.7 mL	1.4 - 2.2	650 µL	1.4 - 2.2	330 µL	1.4 - 2.2	150 µL	1.4 - 2.2	50 µL
Mouse	0.4 - 0.6	1.7 mL	0.4 - 0.6	650 µL	0.4 - 0.6	330 µL	0.4 - 0.6	150 µL	0.4 - 0.6	50 µL

** Do not swirl 96-well plates to distribute cells.*

4) Place the seeded culture vessel in the 37°C incubator and swirl in a figure 8 pattern to evenly distribute the cell suspension. *Do not swirl 96-well plates.*

5) Allow cells to attach for 2-4 hours in a 37°C humidified CO_2 static incubator. Check attachment every hour until sufficient confluency is achieved (the hepatocytes will flatten out over time to fill in the majority of the gaps).

6) After the attachment period, swirl the culture vessel (to suspend the unattached cells) and aspirate media containing non-attached cells.

7) Add appropriate volume of 2-8°C Opti**Culture** Media solution (with or without Opti**Matrix**) to each well or plate and return dishes to incubator.

- Opti**Matrix**, as used for overlay, should be diluted to 0.25 mg/mL in the Opti**Culture** Media.
- To achieve maximum confluency when working with rat hepatocytes, DO NOT include Opti**Matrix** in the Hepatocyte Culture Media at the 2-4 hour time point media change. Opti**Matrix** overlay should be performed 18-24 hours post seeding.

8) Every 24 hours, the media should be aspirated and replaced with Opti**Culture** Media warmed to 37°C.

9) Dosing with compound can begin after the hepatocytes have been in culture for 24-48 hours.

- Media should be aspirated and replaced with fresh dosing solution (Opti**Culture** Media and test compound) at 37°C every 24 hours.
- Cultures can be maintained for 6 to 7 days.