



## Protocol for Thawing and Culturing Cryopreserved Kupffer Cells

The following procedure should 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 Kit (K8700)

This procedure describes the steps required for the isolation of Kupffer cells using OptiThaw Kupffer Cell Thaw / Culture Media.

#### Materials Needed:

- OptiThaw (K8700) - note this media will be used for thawing and culturing Kupffer Cells
- Trypan Blue solution, 0.4%
- 24-well cell culture-treated plate

**Note:** It is important to keep all reagents at 2-8°C during the thawing process. Kupffer cells will adhere to any substrate if the media temperature exceeds 20°C.

- 1) Aliquot 9 mL of cold OptiThaw Kupffer Cell Media (K8700) into a 15 mL conical tube and place on ice.
- 2) Remove the cryo vial from the LN<sub>2</sub> storage unit and immediately place in a 37 ± 1°C water bath for ~80 seconds. The frozen cell pellet should move freely when the cryo vial is inverted. *Do not over-thaw.*
- 3) Dump the frozen pellet from the cryo vial into the 15 mL conical tube containing 9 mL of media. Rinse each cryo vial with 1.0 mL of cold media. Pour this rinse into the 15 mL conical tube. Gently invert the 15 mL conical tube until all ice is melted.
- 4) Centrifuge the cells at 500 x g for 5 minutes at 4°C.
  - Aspirate and discard the supernatant fluid without disturbing the cell pellet.
  - Note that the pellet will be very small
- 5) Gently re-suspend the cell pellet in 500 µL of cold OptiThaw Kupffer Cell Media (4°C) and mix gently with a pipet. (Gently aspirate the media up into the pipet and then dispense back down into the vial 2-3 times.)
- 6) Count the cells and assess viability using the Trypan Blue exclusion assay. Add 10 µL of cell suspension to 10 µL of Trypan Blue solution, 0.4% (Please refer to the attached "Cryopreserved Kupffer Cell Preparation Worksheet).
- 7) Measure the volume of the cell suspension and *q.s.* with OptiThaw Kupffer Cell Media to a final volume that yields 0.5 x 10<sup>6</sup> cells /mL or to the desired density per internal protocol.
- 8) Plate 0.5 mL of cell suspension per well of a 24-well cell culture-treated plate.
- 9) Place the cells in a humidified 37°C/5% CO<sub>2</sub> incubator and swirl in a figure 8 pattern to evenly distribute the cell suspension. (Note that the cells will begin to clump together as the media temperature increases; this is normal)
- 10) Dosing with a test article can begin after the Kupffer cells have been in culture for 24-48 hours even though the cells remain in suspension. Cells will begin to attach at approximately day 5, at which point the media (K8700) can be changed. Prior to this time, a majority of the cells will be in suspension and nutrients could be replenished by addition of warm media (K8700).

# Cryopreserved Kupffer Cell Preparation Worksheet

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

## Kupffer cell Sample Identification

# Vials Thawed	
Sample ID (Lot Number)	1.0 mL

Date of Kupffer cell isolation: \_\_\_\_\_

## Trypan Blue Cell Count Analysis

A trypan blue exclusion analysis should be performed (step 6 in the thawing protocol) following re-suspension of the initial cell pellet.

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

- The dilution factor will equal 2 if a 10 µL aliquot of the cell suspension was dispensed into 10 µL of Trypan Blue solution as stated in the Protocol for Thawing Kupffer Cells for subsequent counting. If an alternate means of dilution was used, a different dilution factor should be calculated.
- The hemacytometer factor will typically equal 10,000. For more information consult your hemacytometer manufacturer.
- Volume of the sample indicates the total volume of the cell suspension from which the counting aliquot was removed.
- The number of viable Kupffer cells may be calculated from the following equation:

$$\frac{A}{\text{quadrants}} \times C \times D \times E \quad \text{where "quadrants" equals the number of quadrants counted on the hemacytometer}$$

- 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 Kupffer cells (determined above)	Desired cell concentration for use (million cells/mL)	Final volume (mL)	Volume of media to add to reach desired conc. (mL)
F	G	H	I

$$H = F/G$$

$$I = H - \text{Volume of sample}$$

## Tips for Working with Kupffer Cells

- It is important to keep all reagents at 2-8°C during the thawing process. Kupffer cells will adhere to any substrate if the media temperature exceeds 20°C.
  - Thaw time is critical; over-thawing cryopreserved Kupffer cells will result in low yield and viability. The solid, frozen pellet should be dumped directly into the 15 mL conical containing 9 mL of cold media.
  - 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.
  - Sekisui XenoTech does not recommend pouring off supernatant, due to the high risk for losing the viable cell pellet during the pour process.
  - Never vortex or vigorously re-suspend the Kupffer cells.
  - We recommend performing two Trypan blue counts after the spin for verification of yield and viability.
  - Although cells begin to attach to the plate surface at day 5, higher confluency will be reached if the cells are allowed to attach for longer periods of time.
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