Protocol for Plating Rat Cryopreserved Kupffer cells and Rat Kupffer-Hepatocyte Co-cultures
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Figure 1—Timeline for plating Kupffer and co-cultures: Kupffer (KC) and Hepatocytes (Heps)

- Plate Kupffer Cells in Plating Media per Protocol
- Change media 4 hrs later with Plating Media
- After KC have been plated for 24 hrs change media into Kupffer Maintenance Media per Protocol
- 1 day Co-culture
- 2 day Co-culture
- 3 day Co-culture
- 4 day Co-culture
- After 24 hours, plate Hepatocytes in Kupffer Plating Media per Protocol
- 4 hours after plating Heps, change media into Co-culture Maintenance Media per Protocol
- Activate KC with lipopolysaccharide (LPS) 24 hrs before dosing. Begin treatment 24 hrs after plating Hepatocytes.

Note: Above timeline for plating Co-cultures assumes plating Kupffer cells the day before plating hepatocytes. Hepatocytes can be plated as soon as 4 hours after Kupffer cells have been plated or as long as 48 hours after Kupffer cells have been plated.

Recommended reagents

**Kupffer Thawing/Plating Media:**

- Advanced DMEM: 500 mL 12491-015
- Hepatocyte Plating Supplement Pack
- Add only Plating cocktail A, and FBS: 1 kit for 500 mL CM3000
  - Do not add Dexamethasone
- Percoll: 1000 mL 17-0891-01
- Dulbecco's Phosphate Buffer Solution (DPBS) 10X: 500 mL 14200-075

**Kupffer Maintenance Media:**

- Advanced DMEM: 500 mL 12491-015
- Hepatocyte Maintenance Supplement Pack
- Add only Maintenance cocktail B: 1 kit for 500 mL CM4000
  - Do not add Dexamethasone
- 2% FBS: 500 mL 16000-044

**Co-Culture Maintenance Media:**

- Advanced DMEM: 500 mL 12491-015
- Hepatocyte Maintenance Supplement Pack (serum-free): 1 kit for 500 mL CM4000
  - Only add Maintenance cocktail, not Dexamethasone
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Multi-well Culture Plates

<table>
<thead>
<tr>
<th>Collage Type 1 - 06 well</th>
<th>Pack of 5</th>
<th>A11428-01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collage Type 1 - 24 well</td>
<td>Pack of 5</td>
<td>A11428-02</td>
</tr>
<tr>
<td>Collage Type 1 - 96 well</td>
<td>Pack of 5</td>
<td>A11428-03</td>
</tr>
</tbody>
</table>

Note: Protocol assumes using 24-well for all seeding densities. Seeding densities for other well formats will need to be adjusted.

Note: Kupffer cell monocultures grow best in media supplemented with at least 2% FBS and NO corticosteroids (e.g. Dexamethasone, Hydrocortisone).

Note: If plating co-culture with hepatocytes, plate Kupffer cells 24 hours prior to plating hepatocytes for best results, however, you can plate hepatocytes 4-48 hours after Kupffer cells are plated.

Protocol

Plating Kupffers from Cryo Vial

1. After receiving the order, store vial in liquid Nitrogen dewar below -130°C (vapor phase).
2. To thaw, remove vial from dewar and thaw in 37°C water bath until only a small sliver of ice is left in the cryo vial.
3. Transfer contents of cryo vial into a 15 mL conical tube on ice, and slowly add 4°C Kupffer Plating Media up to 10 mL volume to dilute sample.  
   **Note:** Kupffer cells are very "sticky" at physiological temperature of 37°C. If media is warmed to 37°C the Kupffer cells will attach to any substrate including walls of conical tube; use of pre-warmed media is not recommend at this step.
4. Centrifuge cells at 500 x g for 5 minutes.
5. Resuspend pelleted cells in 1-2 mL of Kupffer Plating Media using a small serological pipet (1 mL pipet recommended).
6. Count cells using trypan blue exclusion assay.
7. Dilute cells with Kupffer Plating Media to 0.2 x 10⁶ cells/mL (for inflammatory Kupffer/Hepatocyte co-cultures) or to desired density per internal protocol.
8. Plate cells seeding 0.5 mL per well for 24-well format and place cells in humidified 37°C/5% CO₂ incubator and allow them to attach for 4 hrs.
9. After 4 hr attachment, replace media with Kupffer Plating media.
10. After 24 hrs, replace media with Kupffer Maintenance media - Kupffer cell cultures are ready to begin experiment or proceed to next steps to plate a Kupffer cell/Hepatocyte co-culture.  
    **Note:** To maintain Kupffer cell culture, change media into Kupffer Maintenance Media every 24 hours.

Protocol continued on Pg. 4
Protocol for Plating Rat Cryopreserved Kupffer Cells and Rat Kupffer-Hepatocyte Co-cultures

Co-culture with Hepatocytes

11. Seed hepatocytes on top of Kupffer cells following normal protocol for plating rat hepatocytes using Kupffer Plating Media.

   **Note:** Prior to using hepatocytes (Cryopreserved or Fresh), hepatocytes need to be put through a Percoll gradient spin 50:50 v/v of 90% Isotonic Percoll/Kupffer Plating Media. Spin cells at 100 x g for 10 minutes. The g force can be increased to 120 x g if cell recovery is too low. Expect to see an increase in viability and decrease in yield. This step is required to obtain the purest hepatocytes possible in order to establish cytokine base levels following treatment with Lipopolysaccharide (LPS).

   **Note:** 90% Isotonic Percoll is made by diluting Percoll with 10% Dulbecco’s Phosphate Buffer Solution (10X) – Invitrogen Cat No. 14200-075.

   Recommended Rat Hepatocyte Seeding Density:

   - Freshly isolated rat hepatocytes – 24-well plate - 0.6 x 10^6 cells/mL
   - Cryopreserved rat hepatocytes – 24-well plate - 0.8 x 10^6 cells/mL

12. After hepatocytes attach (4-6 hr), change media with Co-culture Maintenance Media.

13. Allow cells to culture for 24 hours prior to conducting experiments.

Kupffer Cell Activation

14. Add lipopolysaccharide (LPS 1ug/mL) in media to activate Kupffer cells prior to experiment (in either Kupffer cell culture or co-culture) to mimic 24 hours inflamed liver state.

   **Note:** LPS activation changes happen fairly quickly, and morphological changes can be seen in less than 2 hr (see Figure 2).

Activated Kupffers – addition of 1ug/ml of LPS to media

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Cell Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-4 hours</td>
<td>Cells start out darker and more square-like, then become more spindly with longer, thin cytoplasmic projections and appear more dendritic-like. Cells exhibit high level of motility by migrating around the plate and digesting nearby debris and other cells.</td>
</tr>
<tr>
<td>4-8 hours</td>
<td>Cells return to a rounder morphology</td>
</tr>
<tr>
<td>8-24 hours</td>
<td>Cell flatten and their cytoplasm enlarges, creating large round cells with many vacoules inside (macrophage like). Cells exhibit lower motility.</td>
</tr>
</tbody>
</table>

Figure 2 — Morphological changes of cryopreserved kupffers seen over time after activation by the addition of Lipopolysaccharide (LPS).

Add LPS → 2-4 hrs → 4-24 hrs

Figure 3 — Co-culture: 1 day on collagen. **Note:** Difficult to see distinct Kupffer Cells when in co-culture with hepatocytes. See red circle below to see Kupffer Cells.