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Hepatocyte and Kupffer Cell (KC) 2D Co-Culture

Instructions for use

Safety Statements

These products are not for use in GMP manufacturing, nor human or animal in vivo use, including use as a diluent or as an excipient, or for diagnostic use.

These products are for research use *only.*

WARNING: LONZA PRIMARY CELLS CONTAIN HUMAN SOURCE MATERIAL; TREAT AS POTENTIALLY INFECTIOUS. Each donor is tested and found non-reactive by an FDA-approved method for the presence of HIV-I, hepatitis B virus and hepatitis C virus. Where donor testing is not possible, cell products are tested for the presence of viral nucleic acid from HIV-1, hepatitis B virus, and hepatitis C virus. Testing cannot offer complete assurance that HIV-1, hepatitis B virus, and hepatitis C virus are absent. All human-sourced products should be handled at the biological safety level 2 to minimize exposure to potentially infectious products, as recommended in the CDC-NIH manual, Biosafety in Microbiological and Biomedical Laboratories, 5th edition. If you require further information, please contact your site safety officer or Scientific Support.

Preparation of Reagents

All work should be performed in a laminar flow hood. Decontaminate the external surfaces of all supplement vials and the medium bottles with 70% ethanol or isopropanol.

Reagents for Hepatocyte: KC Co-Culture

1. **Co-culture Plating Medium**
   Make 20 mL of plating medium by adding the following reagents into a 50 mL conical tube.
   a. 18 mL of DMEM (high Glucose, w/o phenol red and L-Glutamine).
   b. 2mL of FBS (10% final concentration).
   c. 200 µL of 100X MEM non-essential amino acids (NEAA; 1X final concentration).
   d. 200 µL of 100X Insulin-Selenium-Transferrin (ITS-G; 1X final concentration).
   e. 20 µL of 1000X Gentamicin/Amphotericin- (1X GA final concentration).
   f. 400 µL GlutaMAX™ (2X final concentration).

2. **Co-Culture Maintenance Medium**
   Make 50 mL of plating medium by adding the following reagents into a 50 mL conical tube.
   a. 50 mL of DMEM w/o phenol red and L-Glutamine.
   b. 500 µL of 100X MEM NEAA (1X final concentration).
   c. 500 µL of 100X ITS (1X final concentration).
   d. 50 µL of 1000X GA (1X GA final concentration).
   e. 1 mL GlutaMAX™ (2X final concentration).

Reagents for CD68 Immunocytochemistry (ICC)

1. **10% Normal Goat Serum (NGS)**
   a. Add 5 mL of stock Goat Serum to 45 mL of 1X PBS. Scale up or down as needed.
2. **1% Normal Goat Serum**
   a. Using stock Goat Serum: Add 1 mL of stock Goat Serum to 99 mL of 1X PBS.
   b. Using 10% NGS: Add 5 mL of 10% NGS to 45 mL of 1X-PBS.
3. **DAPI Working Solution**
   a. Add 10 mg DAPI to 285 mL DI water for a 100 µM concentration. This intermediate solution can be stored for up to 2 years at -20 °C.
   b. Add 18 µL of DAPI intermediate solution to 6 mL of PBS to make final working solution.

4. **50% Glycerol**
    a. Add 50 mL glycerol stock to 50 mL PBS. Remaining solution can be stored for 1 year at room temperature.

Hepatocyte Preparation

NOTE: All work is to be performed in a laminar flow hood. Handle hepatocytes gently at every step.

1. Warm 50 mL of ready-to-use Hepatocyte Thawing Media in a 37 °C water bath.
2. Thaw hepatocytes at 37 °C for ~2 min until only a sliver of ice remains and pour or dump cells into pre-warmed Hepatocyte Thawing Media from step 1. DO NOT PIPETTE OR VORTEX CELLS.
3. Wash cell vial by pipetting 1 mL Hepatocyte Thawing Media into vial, close vial cap and invert vial a few times. Then, pour the wash back into the 50 mL conical.
4. Centrifuge cells at 100xg for 8 minutes at room temperature.
5. Resuspend pellet in 4 mL of pre-warmed **Co-culture Plating Medium**. Mix cells by gently inverting the conical tube a few times until no cell clumps are visible.
6. Count cells using 0.4% trypan blue and a hemocytometer at a 1:10 dilution by preparing a separate microcentrifuge tube as follows:
    a. Add 200 µL of plating medium.
    b. Add 25 µL of trypan blue.
    c. Add 25 µL of cell suspension.
7. After counting, resuspend cells at 1 x 10^6 cells/mL in pre-warmed Co-culture Plating Medium and gently invert a few times to mix cells.
8. In a 24-well collagen coated plate, add 500 µL of cell suspension to each well (~500,000 hepatocytes per well).

Note: it is recommended to plate, at minimum, duplicate wells for both hepatocyte-only and hepatocyte: KC co-culture for both blank (control) and LPS-treated cultures.

Note: It is recommended to fill empty wells with either PBS or media to prevent evaporation.

9. Incubate cells at 37 °C, 5% CO_2 for 1 hour with gentle rocking every 15 – 20 minutes.

Kupffer Cell Preparation

Note: Kupffer Cells will be added to hepatocyte cultures after the 1 hour incubation in step 9 above. It is recommended to prepare Kupffer Cells 20 – 30 minutes prior to the completion of the 1-hour incubation.

1. Thaw KCs at 37ºC for ~2 min until only a sliver of ice remains and dump cells into 10 mL cold **Co-culture Plating Medium** in a 15 mL conical tube.

Note: Do not warm Co-culture Plating Medium to thaw KCs. KCs will adhere to any substrate at temperature >20ºC.

2. Wash cell vial with 1 mL of Co-culture Plating Medium from 15 mL conical tube and add back to conical tube.
3. Centrifuge cells at 500xg for 5 minutes at 4 °C
4. Resuspend pellet in 1 mL cold plating medium and count using 0.4% trypan blue and a hemocytometer at a 1:5 dilution.
5. After counting, resuspend cells at 500,000 cells/mL in Co-culture Plating Medium. Pipette to mix well.
6. Remove hepatocyte plate from the incubator and place in laminar flow hood.
7. Remove media and unattached cells.
8. Add 250 µL pre-warmed fresh Co-culture Plating Medium to each well.
9. Add 250 µL of KC cell suspension to the same well. This makes a 4:1 hepatocyte: KC co-culture ratio.
    a. A 2:1 hepatocyte: KC ratio may also be used by doubling the KC seeding density. DO NOT change hepatocyte seeding density.
10. Incubate cells at 37 °C, 5% CO_2 for 1 hour, with gentle rocking every 15 – 20 minutes to evenly disperse cells.
11. After incubation, aspirate plating medium with unadhered cells and feed cells with 500 µL pre-warmed **Co-culture Maintenance Medium**.
12. Incubate cells at 37 °C, 5% CO_2 and change media daily with Co-culture Maintenance Medium until day 2.

Note: Co-culture can be incubated for additional days prior to LPS treatment if desired and cells remain healthy.

Co-culture LPS Treatment

1. On day 2 (48hr post-plating), dilute LPS with Co-culture Maintenance Medium at a 1:1000 ratio.
    a. For example, add 3 µL of 1 mg/mL LPS to 2.997 mL of Co-culture Maintenance Medium for a final concentration of 1 µg/mL LPS.
2. Take 500 µL of pre-warmed untreated (control) or LPS-treated Co-culture Maintenance Media and add to each appropriate well.
3. Place cells back into an incubator and incubate for 24 – 48 hours.

4. After incubation, collect all supernatant from each well into each microcentrifuge tube and store at -20°C or -80°C for Enzyme-Linked Immunosorbent Assay (ELISA).

Note: The following steps 5 – 8 may be performed to prepare for CD68 ICC. Disregard steps if assay is not of interest.

5. After supernatant collection, add 400 µL/well of ice cold methanol and incubate at room temperature for 10 minutes to fix/permeabilize cells.
6. After fixing, collect methanol in a conical tube and discard in biohazard trash.
7. Wash cells 2x with 500 µL/well PBS and aspirate.
8. After removing second PBS wash, add 1 mL of 1X PBS to each well, wrap plate in parafilm and store at 4°C until needed for CD68 ICC.

CD68 ICC Preparation

Note: This assay is used to confirm KC presence in the co-culture model. An alternative assay may be used in concert or instead if desired.

1. Remove PBS from fixed cells and add 400 µL 10% NGS for 30 – 60 minutes at room temperature to block cells.
2. During the blocking step, prepare the CD68 antibody and the IgG antibody:
    a. Make 400 µL anti-CD68 antibody per CD68 well by combining 4 µL anti-CD68 antibody per 100 µL 1% NGS and vortex or pipette to mix.
    b. Make 400 µL IgG antibody per IgG well by combining 1µl IgG control antibody per 225 µL 1% NGS and vortex or pipette to mix.
3. After blocking cells, remove the 10% NGS and add 400 µL/well of either IgG or CD68 to appropriate wells.
4. Incubate primary antibodies for 4 hours at room temperature.
5. After incubation, remove primary antibodies and wash 2x with 1 mL PBS per well.
6. Remove second PBS wash and prepare secondary antibody:
    a. Make 400 µL donkey anti-rabbit 568 secondary antibody by combining 1 µL of donkey anti-rabbit 568 per 1000 µL 1% NGS and vortex or pipette to mix.
7. Add 400 µL of donkey anti-rabbit 568 secondary antibody to all wells.
8. Incubate for 1 hour at room temperature in the dark.
9. After incubation, remove secondary antibody and wash 2x with 1 mL PBS per well.
10. Add 400 µL DAPI working solution to each well and incubate for 5 minutes at room temperature.
11. Remove DAPI and wash 2x with 1 mL PBS per well.
12. Remove PBS and add 1 mL 50% glycerol per well.
13. Image using fluorescent microscope.
14. If plate cannot be imaged immediately, wrap plate in parafilm and store at 4°C for up to 1 month until images can be captured.
15. Store plate at -20°C for long-term storage if required for future imaging.

ELISA Preparation

1. Thaw supernatants on ice until completely thawed.
2. Centrifuge supernatants at 13,000xg for 5 minutes at 4°C to pellet debris and dead cells.
3. After centrifugation, prepare samples for ELISA and perform the assay according to the manufacturer's protocol. Avoid touching the bottom of the tube where debris and dead cells are pelleted.
    a. <u>Albumin</u>
        i. Recommended dilution using Abcam Human Albumin ELISA Kit is 1:1000.
    b. <u>IL-6 and TNF-α</u>
        i. Recommended dilution for LPS-treated co-culture samples for IL-6 and TNF-α is 1:50. All other samples may be diluted 1:2 when using the Ella 16x4 cartridge for IL-6 and TNF-α.
        ii. Dilute all samples appropriately prior to loading samples into the Ella cartridge.

Ordering Information

Catalog No.	Description	Size
HUCPI	Cryopreserved Human Hepatocytes	≥ 5 million cells
HLKC-500K	Cryopreserved Human Kupffer Cells	≥ 500 thousand cells
MCHT50	Hepatocyte Thawing Media	50 mL

PBS without Calcium or Magnesium (ThermoFisher Scientific 10010023) mentioned is a product of GIBCO™.

DMEM mentioned (w/ 4.5 g/L Glucose, w/o L-Glutamine and Phenol Red; ThermoFisher 31053028) mentioned is a product of GIBCO™.

MEM Non-Essential Amino Acids (NEAA) Solution 100x (ThermoFisher 11140050) mentioned is a product of GIBCO™.

Insulin-Transferrin-Selenium (ITS-G) 100X supplement (ThermoFisher 41400045) mentioned is a product of GIBCO™.

GlutaMAX™ supplement (ThermoFisher 35050079) is a product of Gibco™

Trypan Blue 0.4% (ThermoFisher 15250061) mentioned is a product of GIBCO™.

DAPI stain (ThermoFisher D1306) mentioned is a product of Invitrogen™.

Collagen I 24-well Clear Flat Bottom TC-treated Plate (Corning® 354408) mentioned is a product from Corning®.

Methanol (Sigma-Aldrich 322415) mentioned is a product from Sigma-Aldrich.

Fetal Bovine Serum (FBS) Premium Grade (VWR 97068-085) mentioned is a product from Avantor® Seradigm.

Recombinant Anti-CD68 antibody (Abcam ab192847) mentioned is a product from Abcam.

Recombinant Rabbit IgG, monoclonal (Abcam ab172730) mentioned is a product from Abcam.

Donkey Anti-Rabbit IgG (Abcam ab175694) mentioned is a product from Abcam.

Goat serum/NGS (Sigma-Aldrich G9023) mentioned is a product from Millipore Sigma.

Glycerol (Sigma-Aldrich G2025) mentioned is a product from Millipore Sigma.

LPS (Sigma-Aldrich L5668-2ML) mentioned is a product from Millipore Sigma.

Human Albumin ELISA Kit (Abcam ab179887) mentioned is a product from Abcam.

Ella™ cartridge 16x4 for IL-6 and TNF-α (ProteinSimple ST01A-PS-003229) mentioned is a product from ProteinSimple.