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Macrophage Polarization and Phagocytosis Assay

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, 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.

1. **KuGM™ Modified Medium**
   a. Add the 50 mL FBS supplement to the 500 mL KuBM™ basal medium as per manufacturer instructions.
   b. Only add 500 µL of the GA-1000 supplement. DO NOT ADD THE WHOLE SUPPLEMENT.
2. **M-CSF Macrophage Differentiation Medium**
   a. Add 1 mL of PBS into M-CSF 10 µg stock vial and mix well (makes 10 µg/mL working solution).
   b. Combine KuGM™ Modified Medium prepared in step 1 + 350 µL M-CSF 10 µg/mL working solution to make 100 mL of 35 ng/mL Macrophage Differentiation Medium.
3. **M1 Polarization Medium**
   a. Prepare this medium on day 6 of culture (see **PART 3: Macrophage Polarization** below).
   b. **IFN-γ 100 µg/mL solution:** Add 0.8 µL 0.1% HSA to 200 µL PBS. Then add this solution to the 20 µg lyophilized vial of IFN-γ.
   c. **LPS 100 µg/mL solution:** Add 10 µL of 1 mg/mL stock LPS solution to 90 µL KuGM™ Modified Medium.
   d. **For each 10 mL of M1 Polarization Medium**: combine 5 µL IFN-γ solution + 1 µL LPS solution + 10 mL of KuGM™ Modified Medium.
4. **M2 Polarization Medium**
   a. Prepare this medium on day 6 of culture (see **PART 3: Macrophage Polarization** below).
   b. **IL-4 100 µg/mL solution:** Add 0.8 µL 0.1% HSA to 200 µL PBS. Then add this solution to the 20 µg lyophilized vial of IL-4.
   c. **IL-10 100 µg/mL solution:** Add 0.4 µL 0.1% HSA to 100 µL PBS. Then add this solution to the 10 µg lyophilized vial of IL-10.
   d. **For each 10 mL of M2 Polarization Medium:** combine 2 µL IL-4 solution + 2 µL IL-10 solution + 10 mL KuGM™ Modified Medium.

5. **Cytochalasin D Medium**
   a. Prepare on day 7 of culture (see **PART 4: Phagocytosis Assay using Live Cell Imaging**).
   b. Reconstitute lyophilized cytochalasin D (1 mg vial) to 4 mM stock solution by adding 493 µL DMSO to the vial and mixing well.
   c. **4 µM Cytochalasin D Medium**: prepare 1 mL of medium by adding 1 µL of 4 mM cytochalasin D stock solution to 1 mL KuGM™ Modified Medium. Scale to total volume needed.

Note about this protocol: There are two possible starting points for the macrophage differentiation protocol.

1. **Part 1** describes the protocol if using cryopreserved PBMCs as the starting material, from which monocytes are first isolated via plastic adherence before being treated with differentiation medium. Upon completing this Part, you would skip Part 2 and move directly to Part 3.
2. **Part 2** describes the protocol if using cryopreserved positively-selected $CD14^+$ Monocytes as the starting material. Skip Part 1 if using Monocytes as the starting material.

Part 1: Macrophage Differentiation Protocol Using PBMCs as Starting Material (Option 1)

NOTE: Perform all work in a laminar flow hood.

NOTE: Cryopreserved cells are delicate and must be thawed and put into culture as quickly as possible with minimal handling. Thaw no more than 2 vials at a time.

NOTE: Follow these steps only if using Peripheral Blood Mononuclear Cells (PBMCs) as a starting material.

1. Add 10 mL of pre-warmed (37°C) KuGM™ Modified Medium to a 15 mL conical centrifuge tube.
2. Thaw PBMC vials (Lonza part no. CC-2702, 50M cells) according to manufacturer instructions in a 37°C water bath for no more than 2 minutes, removing cryo vials once a sliver of ice remains.
   a. Other vialing densities can be used as appropriate to your workflow.
3. Transfer PBMCs to the 15 mL conical tube containing KuGM™ Modified Medium.
4. Centrifuge tubes at 300xg for 10 minutes at room temperature.
5. Remove supernatant and discard.
6. Resuspend cells in 5 mL KuGM™ Modified Medium.
7. Count using trypan blue and a hemocytometer at a 1:100 dilution
   a. Example: First, dilute cells 1:10 KuGM™ Modified Medium (10 µL cells + 90 µL medium, e.g.). Then, dilute again 1:10 in 0.4% trypan blue (e.g., 10 µL diluted cells + 90 µL 0.4% trypan blue).
8. Add KuGM™ Modified Medium to the 15 mL conical tube to resuspend PBMCs at a density of $10x10^6$ cells/mL.
9. Seed cells at the following densities:
   a. For a 24-well plate, add 1 mL of cell suspension for a total of $10x10^6$ cells/well.
   b. For a 96-well plate, add 100 µL of cell suspension for a total of $1x10^6$ cells/well.
10. Incubate in a humidified incubator for 2 hours at 37°C, 5% CO_2.
11. Remove plate from incubator, swirl to gently remove non-adhered cells, and aspirate media from all wells.
12. Wash plate 2x with pre-warmed (37°C) KuGM™ Modified Medium.
13. Add M-CSF Macrophage Differentiation Medium to each well:
    a. For a 24-well plate: 1 mL per well
    b. For a 96-well plate: 100 µL per well
14. Replace plate in the incubator. **Do not disturb until day 3**.
15. **PROCEED TO PART 3.**

Part 2: Macrophage Differentiation Protocol Using Positively-selected $CD14^+$ Monocytes as Starting Material (Option 2)

NOTE: Perform all work in a laminar flow hood.

NOTE: Cryopreserved cells are delicate and must be thawed and put into culture as quickly as possible with minimal handling. Thaw no more than 2 vials at a time.

NOTE: Follow these steps only if using positively-selected $CD14^+$ Monocytes as a starting material.

1. Add 10 mL of pre-warmed (37°C) KuGM™ Modified Medium to a 15 mL conical centrifuge tube.
2. Thaw $CD14^+$ Monocytes (Lonza part no. 2W-400B; positively-selected) according to manufacturer instructions in a 37°C water bath for no more than 2 minutes, removing cryo vials once a sliver of ice remains.
3. Transfer monocytes to the 15 mL conical tube containing KuGM™ Modified Medium.
4. Centrifuge tubes at 300xg for 10 minutes at room temperature.

5. Remove supernatant and discard.
6. Resuspend cells in 5 mL KuGM™ Modified Medium.
7. Count using trypan blue and a hemocytometer at a 1:20 dilution
   a. Example: 10 µL cells + 190 µL 0.4% trypan blue
8. Add M-CSF Macrophage Differentiation Medium to the 15 mL conical tube to resuspend $CD14^+$ Monocytes at a density of $1x10^6$ cells/mL.
9. Seed cells at the following densities:
   a. For a 24-well plate, add 1 mL of cell suspension for a total of $1x10^6$ cells/well.
   b. For a 96-well plate, add 100 µL of cell suspension for a total of $0.1x10^6$ cells/well.
10. Incubate in a humidified incubator at 37°C and 5% CO_2. **Do not disturb until day 3.**
11. **PROCEED TO PART 3.**

Part 3: Macrophage Polarization

1. On day 3, change media by carefully aspirating medium from all wells and replacing with the appropriate amount of M-CSF Macrophage Differentiation Medium:
   a. For a 24-well plate: 1 mL per well
   b. For a 96-well plate: 100 µL per well
2. On day 6, make M1 and/or M2 Polarization Medium as described in the "Preparation of Reagents" section of this protocol.
3. Carefully aspirate medium from each well and discard.
4. For polarization, add the appropriate polarization medium (M1, M2, or naïve control [M-CSF Macrophage Differentiation Medium]) to each well:
   a. For a 24-well plate: 1 mL per well
   b. For a 96-well plate: 100 µL per well
5. Return to the incubator and culture for 24 hours.

NOTE: After 24 hours, polarization should have occurred (to M1 or M2 state, depending on which polarization medium was used). A variety of analyses or assays may be carried out at this point. Proceed to **PART 4** for an example of a phagocytosis assay using a live cell imaging system.

Part 4: Phagocytosis Assay Using Live Cell Imaging

NOTE: We used a Sartorius Incucyte® SX5 Live Cell Analysis Instrument to carry out this assay. Other systems may be used, but may require some optimization of instrument parameters and staining protocol.

NOTE: We performed this assay in a clear, 96-well flat-bottomed plate format using cytochalasin D as a phagocytosis inhibitor. Other well formats and compounds can be tested, adjusting for volume and concentration, following the same general workflow as below.

1. Reconstitute pHrodo™ bioparticles: On day 7 (24 hours after addition of M1/M2 Polarization Medium), **2 hours prior to establishing the assay**, reconstitute pHrodo™ green *E. coli* bioparticles (2 mg vial) by adding 2 mL PBS, transferring mixture to a glass vial, and sonicating in a water bath for 15 minutes. Return to the refrigerator until adding to cells.

NOTE: Reconstituted pHrodo™ bioparticles can be stored at 4°C for up to one month.

2. Prepare **cytochalasin D medium** as described in the "Preparation of Reagents" section above.
3. **1 hour prior to assay start**, remove polarization media from all wells.
4. Wash all wells 1x with KuGM™ Modified Medium.
5. Add 100 µL of KuGM™ Modified Medium to all wells that will serve as assay blanks (no pHrodo™ bioparticles, no treatment).
6. Add 75 µL of KuGM™ Modified Medium to all treatment wells with pHrodo™ bioparticles.
7. Add 50 µL of KuGM™ Modified Medium and 25 µL of 4 µM cytochalasin D medium (final concentration 1 µM per well) to negative control wells. Incubate for 1 hour at 37°C and 5% CO_2.
8. **30 minutes prior to assay,** sonicate the pHrodo™ bioparticles in a water bath again for 15 minutes.
9. Dilute the pHrodo™ bioparticle suspension to 0.4 µg/µL by adding 1 mL of the pHrodo™ bioparticle suspension to 1.5 mL of KuGM™ Modified Medium in a separate glass vial.
10. Sonicate the diluted pHrodo™ bioparticle suspension for 10 minutes in a water bath.
11. Vortex the diluted pHrodo™ bioparticle suspension after dilution. Do this immediately prior to adding to wells.
12. Add 25 µL of diluted pHrodo™ bioparticle solution to all control and treatment wells (defined in steps #6 and #7 above). All wells now contain 100 µL volume.
13. Immediately place into the incubator with live cell imager and initiate image capture protocol.
    a. We recommend 20x magnification
    b. Color channel should be set to the appropriate fluorescent signal for the fluorescent tag used on the bioparticle (e.g., GFP green). Consult bioparticle manufacturer instructions for more information.

c. 3x images per well recommended
d. The Incucyte® protocol used, captures 3 images per well every 15 minutes for 6 hours, then every 1 hour for the next 18 hours.
e. Total assay time = 24 hours.

If performing the assay described above, macrophages (especially M2 macrophages) treated with green GFP-tagged pHrodo™ bioparticles should begin to fluoresce as they consume the bioparticles over time, resulting in a stronger fluorescent signal and should appear visually greener in the composite videos. Macrophages treated with cytochalasin D and pHrodo™ bioparticles should show inhibited rates of consumption and far lower fluorescence. Macrophages not treated with fluorescent bioparticles should show no fluorescence at all.

Example videos can be found on the Lonza website.

Ordering Information

Catalog No.	Description	Size
CC-2702	Cryopreserved Human Peripheral Blood Mononuclear Cells (HPBMC)	≥50 million cells
CC-2703		≥100 million cells
CC-2705		≥25 million cells
CC-2704		≥10 million cells
2W-400A	Cryopreserved Human Peripheral Blood $CD14^+$ Monocytes, Positively Selected	≥40 million cells
2W-400B		≥20 million cells
2W-400C		≥10 million cells
MKC-500BK	KuGM™ Kupffer Cell Culture BulletKit®	500 mL Basal medium + supplement kit

Corning® 96-Well plate (Corning® 3595) mentioned is a product of Corning®.

Corning® Falcon® 24-Well plate (Corning® 353047) mentioned is a product of Corning®.

Gibco® Trypan Blue 0.4% (ThermoFisher Scientific 15250061) mentioned is a product of GIBCO®.

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

Invitrogen™ pHrodo™ Green *E. coli* BioParticles™ Conjugate for Phagocytosis (FisherScientific P35366) mentioned is a product of Invitrogen™.

Cytochalasin D (Sigma C8273-1MG) mentioned is a product of MilliporeSigma.

Recombinant Human M-CSF (PeproTech 300-25) mentioned is a product of PeproTech.

IFN-γ (PeptroTech 300-02) mentioned is a product of PeproTech.

LPS (Sigma L-5668) mentioned is a product of MilliporeSigma.

IL-4 (PeproTech 200-04) mentioned is a product of PeproTech.

IL-10 (PeproTech 200-10) mentioned is a product of PeproTech.

Incucyte® SX5 Live Cell Analysis Instrument mentioned is a product from Sartorius.]

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