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Dendritic Cell (DC) and T Cell Assay from Matched PBMCs

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.

1. **Release Buffer**
   Prepare a stock solution of 5 mM EDTA in PBS without Ca^{2+} or Mg^{2+} by aseptically adding 10 mL of 0.5 M EDTA to 990 mL PBS. This buffer can be stored at 2-8°C for 1 year.
2. **Adherence Medium**
   Supplement X-VIVO® 15 Serum-free Hematopoietic Cell Medium with 0.5% heat inactivated human plasma containing 50 U/mL Heparin. Keep medium at 4°C.
3. **DC Culture Medium Base**
   Prepare culture medium base during incubation for plastic adherence. Do not add cytokines to culture medium base more than 30 minutes prior to completion of plastic adherence incubation. Add the following cytokines to the X-VIVO® 15 Serum-free Hematopoietic Cell Medium at the indicated final concentrations: IL-4 (1000 U/mL) and GM-CSF (800 U/mL). Once cytokines are added to medium do not store medium for longer than 30 minutes. Medium supplemented with cytokines should be made fresh each time prior to use.
4. **DC Maturation Medium with KLH Antigen**
   On Day 3 of DC culture, prepare DC Culture Medium Base and add 0.23 U/mL Heparin. Add Keyhole Limpet Hemocyanin (KLH) to the medium for a final concentration of 50 µg/ml.
5. **DC Maturation Medium without KLH Antigen**
   On Day 3 of DC culture, prepare DC Culture Medium Base and add 0.23 U/mL Heparin.
6. **IL-2 Medium**
   Prepare IL-2 working solution by diluting IL-2 (100 µg/mL) into sterile PBS without Ca^{2+} or Mg^{2+} at a 1:20 dilution to make a working solution of IL-2 (5 µg/mL). Prepare stock IL-2 medium by adding 1 µL of IL-2 (5 µg/mL) working solution to 1 mL X-VIVO® 15 for a final concentration of 5 ng/mL.

Culturing and Maturation of DCs

NOTE: All work is to be performed in a laminar flow hood.

1. Pipette pre-warmed X-VIVO® 15 Serum-free Hematopoietic Cell Medium into a 50 mL conical tube. 25-30 mL is recommended for Lonza PBMCs (CC-2703; 100 million cells). Adjust the volume according to cell number.
2. Thaw PBMCs in a water bath at 37°C for 1.5 - 2 min and pipette cells into the X-VIVO® 15 Serum-free Hematopoietic Cell Medium in the 50 ml conical tube from step 1. A sliver of ice should remain in the amp when transferring to the conical tube.
3. Wash cell vial with 1 mL X-VIVO® 15 Serum-free Hematopoietic Cell Medium and add wash to 50 mL conical tube from step 2.
4. Centrifuge cells at 300xg for 10 minutes at room temperature.
5. Aspirate supernatant without disturbing cell pellet and resuspend pellet in 1-3 mL X-VIVO® 15 Serum-free Hematopoietic Cell Medium. Count the cells using Trypan Blue 0.4% and a hemocytometer.
6. After counting, plate PBMCs at $5x10^6$ cells/mL in adherence medium into appropriate flask or well-plate.
7. Incubate the flasks/wells/plates at 37°C ± 2°C , 5% CO_2 ± 2% CO_2 for 2 hours for adherence. Verify adherence of cells under the microscope. Cells should not be free floating or move with the media as the plate is inspected under the microscope.
    a. **Note:** About 10-30% of cells will adhere.
8. After 2 hours, aspirate off the non-adherent cells. Rinse the adherent cells by gently adding pre-warmed PBS without Ca^{2+}, Mg^{2+} or EDTA without disturbing attached cells. Gently tap flask and swirl wash around the flask, followed by immediate aspiration of non-adherent cells.
9. Repeat wash step once.
10. Feed adherent cells with DC Culture Medium Base containing cytokines. Adjust volume of culture medium base containing cytokines according to flask or well-plate format. Place the flasks/plates at 37°C ± 2°C, 5% CO_2 ± 2% CO_2 for 72 hours.
11. On day 3, collect medium and nonadherent cells and spin at 300xg for 10 minutes.

Note: Pre-isolated human dendritic cells (Cat: CC-2701) are available for purchase from Lonza. If using Lonza dendritic cells, start protocol from Step 12. Use of pre-isolated Lonza dendritic cells result in a reduction of time of 3 days.

12. Resuspend cell pellet in DC Maturation Media with KLH Antigen and reseed evenly in the flask or well-plate.
13. Add Lipopolysaccharide (LPS) at 1 µL/mL.
14. Incubate the flasks/plates with KLH Antigen at 37°C ± 2°C, 5% CO_2 ± 2% CO_2 for 6 hours.
    a. Note: If it is desirable to have DCs that do not incorporate the KLH Antigen, the cell pellet from step 12 can be prepared with DC Maturation Media without KLH Antigen and reseeded evenly in the flask or well-plate. Add LPS at 1 µL/mL. Place the flasks at 37°C ± 2°C, 5% CO_2 ± 2% CO_2 for 72 hours.
15. Once the DCs are exposed to KLH for 6 hours, collect media and nonadherent cells and spin at 300xg for 10 minutes.
16. Resuspend pellet in DC Maturation Media **without KLH Antigen** and reseed evenly back into culture.
17. Add 1 µL/mL LPS to each culture. Place the flasks/plates at 37°C ± 2°C, 5% CO_2 ± 2% CO_2 for 72 hours.
    a. See **Figure 1** for mature DC morphology
18. Prior to harvest on day 6, ensure that release buffer is ice cold. Remove culture medium and any unattached cells from each flask.
19. Briefly rinse flasks with release buffer. Collect rinse in conical tube.
20. Add additional release buffer to each flask and incubate at room temperature for 10 minutes.

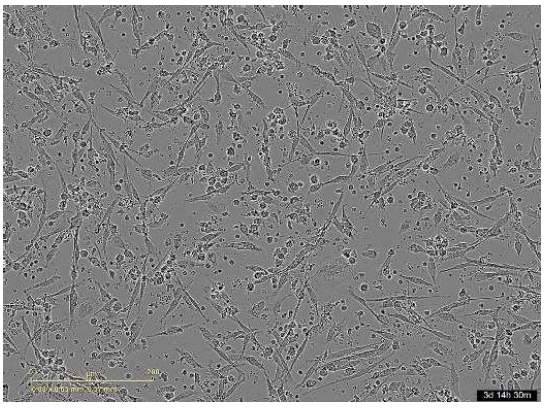

Figure 1: Mature DC morphology after 24 hour exposure to LPS. Mature DCs should have a rough surface with multiple pseudopodia.

21. After incubation, blast off attached cells by tapping sides of flask/well-plate and by pipetting release buffer up and down over cells several times to assist with detachment. Repeat rinse with release buffer one additional time. Remove all detached cells and place them in a conical tube. Centrifuge cells at 200xg for 15 minutes.
22. Resuspend cells in 1 mL IL-2 medium.
23. Count cells via hemocytometer using Trypan Blue 0.4%.
24. Resuspend cells at a concentration of $1.6x10^6$ cells/mL or alternatively at a concentration more appropriate for your experimental setup.
    a. Note: We recommend that DCs should be seeded at a 1:10 ratio to CD8+ T cells.
25. We recommend assessing the dendritic cell population via immunophenotyping to confirm maturation as well as purity of the cells. This can be done on days 0, 3, and 6 of the DC culture. Markers that may be used include CD14, CD1a, CD80, CD83, CD86, and HLA-DR.

DC: T Cell Assay

NOTE: All work is to be performed in a laminar flow hood.

1. Pipette 22.5 mL of pre-warmed X-VIVO® 15 Serum-free Hematopoietic Cell Medium into a 50 mL conical tube.
2. Thaw 1 ampule of Lonza PBMCs (CC-2703; 100 million cells) at 37°C for ~2 min and pipette cells into X-VIVO® 15 Serum-free Hematopoietic Cell Medium in the 50 mL conical tube from step 1.
    a. Note: Other Lonza PBMC products can be used here as well. Adjust volume of DNAse used based on total number of cells to match ratio described here.
3. Wash cell vial with 1 mL X-VIVO® 15 Serum-free Hematopoietic Cell Medium and add wash to conical tube.
4. Add DNAse in PBS to a final concentration of 100 µg/mL (e.g., 2.72 mL of 1 mg/mL DNAse in PBS).
5. Incubate at room temperature for 15 minutes.
6. Centrifuge cells at 300xg for 10 minutes at room temperature.
7. Aspirate supernatant without disturbing cells and resuspend pellet in 5-10 mL PBS. Count using Trypan Blue and a hemocytometer.
8. Purify CD8+ T cells using CD8 MicroBeads by Miltenyi Biotec and following manufacturer's protocol.
9. Once purified, resuspend cells in IL-2 Medium and count using Trypan Blue 0.4% and a hemocytometer.

Note: Pre-isolated human CD8+ T cells (Cat: 2W-300) are available for purchase from Lonza. If using Lonza CD8+ T cells, thaw the cells as described on the Lonza website and proceed with protocol from Step 10. Use of Lonza CD8+ T cells results in a reduction of reagents and time.

10. Resuspend CD8+ T cells at 1.6×10^7/mL in IL-2 medium or alternatively at a concentration more appropriate for your experimental setup.
    a. Note: We recommend that DCs should be seeded at a 1:10 ratio to CD8+ T cells.
11. Prepare assay plates. We suggest 1 assay plate for each of your endpoints (see steps 13 and 14 for recommended endpoints). Add T cells to each well (final concentration of $4x10^5$ cells/well in a 48-well plate – adjust according to tissue plate size). Add DCs to each well (final concentration of $4x10^4$ cells/mL in a 48-well plate – adjust according to tissue plate size). For a 48-well plate, the following volumes and controls are recommended:
    a. Negative control wells: 475 µL IL-2 Medium.
    b. Positive control wells: 462.5 µL IL-2 Medium + 25 µL CD8+ T Cells (1.6×10^7 / mL) + 12.5 µL of ImmunoCult™ CD3/CD28 antibody from StemCell™ Technologies.
    c. DC and T cells with IL-2 wells: CD8+ T cells with mature DCs (exposed to KLH) at a ratio 10:1 (T cell: DC); e.g., 25 µL T cells (1.6×10^7 cells/mL) + 25 µL mature DCs (1.6×10^6 cells/mL) in 450 µL IL-2 medium.
12. Allow assay to incubate for 7 days at 37°C, 5% CO_2.
13. We recommend an assessment of T cell population via immunophenotyping at days 0 and 7 to confirm activation of T cells. This may include markers for T cell population such as CD3 and CD8 and activation markers such as CD25 and CD69.
14. Additionally, T cell proliferation can be assessed at days 0 and 7 via Click-iT™ EdU (e.g., Click-iT™ Plus EdU AlexaFluor™ 488 Flow Cytometry Assay Kit) by Thermo Fisher Scientific. We recommend following the manufacturer's protocol with an EDU incubation time of 2 hours.

Ordering Information

Catalog No.	Description	Size
CC-2702, CC-2703, CC-2705	Human Peripheral Blood Mononuclear Cells (HPBMC), Cryopreserved	≥ 50 million cells, ≥100 million cells, ≥25 million cells
CC-2701	NHDC – Human Dendritic Cells	≥ 2.5 million cells
2W-300	CD8+ cytotoxic T cells from PBMCs	≥ 10 million cells
04-418Q, 02-060Q (EU and ROW)	X-VIVO® 15 Serum-free Hematopoietic Cell Medium	1 L, complete with L-Glutamine, gentamicin, and phenol red; xenofree

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

EDTA (Fisher Scientific BP2482100) mentioned is a product of Fisher Scientific.

Heparin (Fresenius Kabi USA 504015) mentioned is a product from Fresenius Kabi.

IL-4 (R&D Systems 204-IL-010) mentioned is a product from R&D Systems.

GM-CSF (PeproTech 300-03) mentioned is a product from PeproTech.

Keyhole Limpet Hemocyanin (KLH; ThermoFisher Scientific 77600) mentioned is a product of ThermoFisher Scientific.

IL-2 (R&D Systems 202-IL-500) mentioned is a product from R&D Systems.

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

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

DNAse (Worthington Biochemical LS002007) mentioned is a product of Worthington Biochemical.

CD8 MicroBeads, human (Miltenyi Biotec 130-045-201) mentioned is a product from Miltenyi Biotec.

ImmunoCult™ CD3/CD28 antibody (StemCell™ Technologies 10991) is a product from StemCell™ Technologies.

Click-iT ™ Plus EdU AlexaFluor™ 488 Flow Cytometry Assay Kit (ThermoFisher Scientific C10632) mentioned is a product from ThermoFisher Scientific.