SARTORIUS

Protocol

Detailed T Cell Incucyte® Transendothelial Migration Chemotaxis Protocol

Materials

- Freshly isolated blood
- Lympholyte®-H Cell Separation Media (Cedarlane CL5010)
- Dynabeads® Human T-activator CD3/CD28 (Life Technologies 11131D)
- RPMI 1640 Medium (Life Technologies 11875-085)
- Dialyzed Fetal Bovine Serum (FBS; Omega Scientific FB-03)
- HUVEC Cells(LONZA C2517A)
- EGM-2 MV Bullet Kit (LONZA CC-3202)
- Collagen Type I Rat Tail (BD Biosciences 354236)
- Fibronectin (Sigma Aldrich F1141)
- Acetic Acid (0.02N) — used in Collagen coating step
- D-PBS -/- (w/o Ca^{2+}, Mg^{2+}, Life Technologies 10010)— used in Fibronectin coating step
- D-PBS +/+ (with Ca^{2+}, Mg^{2+}, Life Technologies 14040)— used in monolayer wash step
- SDF-1a (R&D systems 350-NS-050)
- Incucyte® Clearview 96-well Plate (Sartorius Cat. No. 4582, 4599, or 4648)

Create HUVEC Monolayer (Day 1)

1. Prepare extracellular matrix coating of either 50 µg/mL Collagen diluted with 0.02N Acetic Acid or 5 µg/mL Fibronectin diluted with D-PBS (-/-).
2. Using reverse pipetting, add 150 µL of coating solution into the reservoir of an Incucyte® Clearview 96-well Plate. Gently place the insert into the reservoir and reverse pipette 20 µL of the fibronectin or collagen solution into the insert an Incucyte® Clearview Plate.
3. Incubate for 1 hour at ambient temperature.
4. During incubation, harvest and count HUVEC cells and prepare a cell seeding stock of 100,000 cells/mL in full growth medium (EGM-2).
5. Aspirate the coating from the reservoir plate and replace with 200 µL of D-PBS (-/-) and gently return the insert into the reservoir plate.
6. To the insert, directly add 60 µL D-PBS (-/-) to the inserts containing coating, then aspirate the entire volume.
7. Immediately seed 60 µL of the HUVEC seeding stock using a multi-channel pipette and reverse pipetting technique into every well of the insert plate (60 µL per well, 6,000 cells per well).

Calculation: 100,000 cells/mL x 0.06 mL = 6,000 cells per insert well.

8. Allow the HUVECs to settle at ambient temperature on a level surface for 15 minutes.
9. Place the Incucyte® Clearview Plate containing HUVEC cells at 37 °C and incubate for 24 hours.

Initiating TEM (Day 2)

1. After HUVEC monolayer has formed, gently wash the monolayer 2X with D-PBS (+/+), using partial washes

   Note: It is important not to disrupt the HUVEC monolayer. It is recommended to gently remove about half of the growth medium then add 60 µL D-PBS for both washes. At the final wash step, remove as much of the medium/D-PBS as possible without disrupting the monolayer.
2. Prepare T cell seeding stock at 83,333 cells/mL in defined EBM-2 (EBM media + 2% FBS + hydrocortisone + ascorbic acid + heparin).
3. Using a manual multi-channel pipette and reverse pipetting technique, seed cells (60 µL per well, 5,000 cells per well) into every well of the insert plate of an Incucyte® Clearview Plate, being careful not to disrupt the HUVEC monolayer.

   Calculation: 83,333 cells/mL x 0.06 mL = 5,000 cells per insert well.
4. Centrifuge the Incucyte® Clearview Plate for 3 minutes at 50 x g in order to quickly bring the T cells to the monolayer surface. Alternatively, if centrifugation is not possible, allow the T cells to settle on the endothelial monolayer at ambient temperature for 45–60 minutes.
5. Using a manual multi-channel pipette, add 200 µL of the chemoattractant and control medium to the appropriate wells of the second Incucyte® Clearview Reservoir Plate.
6. Carefully transfer the insert plate containing the cells into the pre-filled second reservoir plate containing assay medium ± chemoattractant.
7. Place the Incucyte® Clearview Plate into the Incucyte® Live-Cell Analysis System and allow the plate to warm to 37 °C for at least 15 minutes.

   Note: After 15 minutes, wipe away any condensation that remains on the outside of the plate lid or bottom of the reservoir.
8. In the Incucyte® software, schedule 24 hour repeat scanning (10X) for every 30 minutes.

   Note: This schedule is only for a scanning a single plate. Fewer scans will be required if scheduling multiple plates.

   a. Objective: Ensure 10X objective is installed
   b. Vessel Type: Select "Incucyte® Clearview Plate"
   c. Channel Selection: Select "Phase"
   d. Scan Mode: Select "Chemotaxis (Top/Bot)" scan type and desired Scan Pattern
   Note: The Incucyte® Live-Cell Analysis System estimates a scan time of 20 minutes per plate (phase only); however, the actual scan time can take longer.

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