SARTORIUS

Protocol

Incucyte® Chemotatic Transendothelial Migration Assay

For Quantification of Leukocyte Migration Across an Endothelial Monolayers

This protocol is intended for the measurement of transendothelial migration by leukocytes. This method utilizes the Incucyte® Clearview 96-Well Plate and the Incucyte® Live-Cell Analysis System for imaged-based measurements of diapedesis.

Required Materials

- Leukocytes (e.g., activated primary T cells or neutrophils)
- Endothelial cells (e.g., HUVEC or EA.hy9226)
- Endothelial growth medium
- Assay medium
- Collagen Type I Rat Tail (BD Biosciences Cat. No. 354236) or
- Fibronectin (Sigma Aldrich Cat. No. F1141)
- Acetic Acid
- D-PBS -/- (w/o Ca^{2+}, Mg^{2+}, Life Technologies Cat. No. 10010)—used in Fibronectin coating step
- D-PBS +/+ (with Ca^{2+}, Mg^{2+}, Life Technologies Cat. No. 14040)—used in monolayer wash step
- Incucyte® Clearview 96-Well Plate (Sartorius Cat. No. 4582 or 4599)

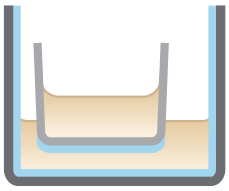
General Guidelines

The Incucyte® Live-Cell Analysis System relies on images to process data; thus, it is important to avoid bubbles and follow our protocol recommendations to achieve superior assay performance and imaging. We recommend the following techniques to eliminate bubbles from your experiment:

- Reverse-pipette at the coating step and when adding cells to the insert. Reverse pipetting reduces the risk of splashing or bubble formation. In reverse pipetting, the volume aspirated into the tip is larger than the volume delivered to the receiving vessel.
  - Press the plunger to the second stop.
  - Dip the pipette-tip into the solution.
  - Release the plunger until the starting has been reached.
  - Move the pipette-tip to the receiving vessel.
  - Dispense the liquid by pressing the plunger to the first stop. Some liquid will remain in the tip.
  - Repeat second to fifth steps throughout the plate.
- Triturate with an additional cell volume or reduced volume setting (e.g., 60 µL cell volume added, mix by reverse-pipetting up and down with 30 µL) to dislodge bubbles that may have been trapped at the membrane-insert interface. Perform this immediately after cell addition.
- Remove bubbles at the liquid surface by gently squeezing a wash bottle containing 100% ethanol, with the inner straw removed, to blow vapor over the surface of each well.

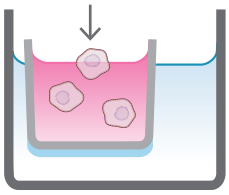
Protocol

1. Coat insert



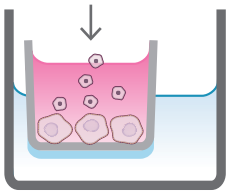
Coat membrane with extracellular matrix (ECM): 20 µL (insert side) and 150 µL (reservoir side).

2. Seed endothelial cells



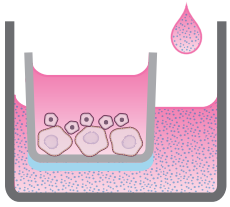
Seed endothelial cells (60 µL/well, 6,000 cells/mL) into the Incucyte® Clearview insert. Allow monolayer to form overnight.

3. Seed leukocytes



Seed leukocytes (60 µL/ well, 5,000 cells/mL) onto endothelial monolayer.

4. Add chemoattractant



Add 200 µL of chemoattractant or controls to reservoir plate. Place the insert into the prefilled reservoir plate and image in Incucyte® Live-Cell Analysis System.

Day 0

Coat Insert

1.1 Prepare extracellular matrix coating of either 50 µg/mL collagen diluted with 0.02 N acetic acid or 5 µg/mL fibronectin diluted with D-PBS (-/-).

1.2 Aliquot 150 µL of coating solution into the reservoir. Gently place the Incucyte® Clearview insert into the Incucyte® Clearview reservoir and pipette 20 µL of the fibronectin, or collagen, solution into the insert.

1.3 Incubate for 1 hour at ambient temperature.

Create Endothelial Cell Monolayer

2.1 During incubation, harvest and count endothelial cells and prepare a cell seeding stock of 100,000 cells/mL in full growth medium.

2.2 Aspirate the coating from the reservoir plate and replace with 200 µL of D-PBS (-/-) and gently return the insert into to the reservoir plate.

2.3 To the insert, directly add 60 µL D-PBS (-/-) to the inserts containing coating, then aspirate the entire volume.

2.4 Immediately seed 60 µL of the endothelial seeding stock in growth medium using a multi-channel pipette 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.

2.5 Allow the cells to settle at ambient temperature on a level surface for 15 minutes.

2.6 Place the Incucyte® Clearview Plate containing cells at 37° C and incubate for 24 hours.

Day 1

Seed Leukocytes

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

NOTE: It is important not to disrupt the 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.

3.2 Prepare leukocyte cell seeding stock at 83,333 cells/mL in appropriate assays medium.

3.3 Using a manual multi-channel pipette and reverse pipetting technique, seed 60 µL of the leukocytes seeding stock (5,000 cells per well) into every well of the insert plate, being careful not to disrupt the endothelial monolayer.
Calculation: 83,333 cells/mL x 0.06 mL = 5,000 cells per insert well.

3.4 Centrifuge the Incucyte® Clearview Plate for 3 minutes at 50 x g in order to quickly bring the leukocytes to the monolayer surface. Alternatively, if centrifugation is not possible, allow the leukocytes to settle on the endothelial monolayer at ambient temperature for 45–60 minutes.

Add Chemoattractant

4.1 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.

4.2 Carefully transfer the insert plate containing the cells into the pre-filled second reservoir plate containing assay medium ± chemoattractant.

4.3 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. After 15 minutes, wipe away any condensation that remains on the outside of the plate lid or bottom of the reservoir.

4.4 In the Incucyte® integrated software, schedule 24 hour repeat scanning (10X) for every 30 minutes.
   a. Scan Type: Chemotaxis
   b. Objective: 10X
   c. Channel selection: Phase Contrast + Fluorescence channel if leukocytes are fluorescently labeled

Leukocyte	Endothelial Cells	Endothelial Growth Medium	Assay Medium	Coating (20 µL Top/150 µL Bottom)	Seeding Density Endothelial: Leukocyte (Cells/Well)
Jurkat with	HUVEC	EGM-2	EBM-2 + 2% FBS with # supplements	5 µg/mL fibronectin or 50 µg/mL collagen	6,000 : 5,000
Primary T Cells	HUVEC	EGM-2	EBM-2 + 2% FBS with # supplements	5 µg/mL fibronectin or 50 µg/mL collagen	
	EA.hy926	DMEM + 10% dialyzed FBS	RPMI + 0.5% dialyzed FBS	50 µg/mL collagen	
Neurophils with	HUVEC	EGM-2	EBM-2 + 2% FBS with # supplements	5 µg/mL fibronectin or 50 µg/mL collagen	

supplement includes gentamycin, hydrocortisone, and ascorbic acid

Find more information at www.sartorius.com/incucyte

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Sales and Service Contacts

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Essen BioScience, A Sartorius Company
www.sartorius.com/incucyte
E-Mail: AskAScientist@sartorius.com

North America
Essen BioScience Inc.
300 West Morgan Road
Ann Arbor, Michigan, 48108
USA
Telephone +1 734 769 1600
E-Mail: orders.US07@sartorius.com

Europe
Essen BioScience Ltd.
Units 2 & 3 The Quadrant
Newark Close
Royston Hertfordshire
SG8 5HL
United Kingdom
Telephone +44 1763 227400
E-Mail:
euorders.UK03@sartorius.com

APAC
Essen BioScience K.K.
4th Floor Daiwa Shinagawa North Bldg.
1-8-11 Kita-Shinagawa
Shinagawa-ku, Tokyo
140-0001
Japan
Telephone: +81 3 6478 5202
E-Mail: orders.US07@sartorius.com

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