PROTOCOL

IncuCyte® Angiogenesis PrimeKit Assay

For the fluorescent detection of vascular tube formation and disruption

This protocol describes a solution for measuring both positive and negative effects on the formation of vascular networks using human endothelial cells co-cultured with human dermal fibroblasts. The IncuCyte Angiogenesis PrimeKit Assay demonstrates all phases of the angiogenesis process, including proliferation, migration, and, eventually, differentiation and anastomosis to form complex angiogenic networks. In conjunction with the IncuCyte® Live-Cell Analysis System, the IncuCyte PrimeKit can be used to kinetically study vascular network response to inhibitors and stimulators of angiogenesis.

Required materials

- IncuCyte® Angiogenesis 96-well PrimeKit (Essen Bioscience Cat# 4452)
  1. Dry Ice Shipment:
     a. Normal Human Dermal Fibroblast (NHDF)
     b. Human Umbilical Vein Endothelial Cells - CytoLight Green (HUVEC CytoLight Green)
     c. Seeding Media Supplement (2 mL total volume)
     d. Growth Media Supplement (0.4 mL total volume)
     e. Assay Media Supplement (2.5 mL total volume)
     f. Growth Factor/Suramin Supplement Kit (if ordered)
  2. Room Temperature Shipment:
     a. Seeding Media (40 mL total volume)
     b. Growth Media (20 mL total volume)
     c. Assay Media (125mL total volume)
     d. 96-well Assay Plate

Additional optional materials

- IncuCyte® Angiogenesis PrimeKit VEGF/Suramin Supplement Kit (Essen Bioscience Cat# 4437)
- IncuCyte® Angiogenesis PrimeKit bFGF/Suramin Supplement Kit (Essen Bioscience Cat# 4438)
- IncuCyte® PrimeKit Optimized Assay Medium (Essen Bioscience Cat# 4541)
- Test treatments of interest

General Guidelines

- Remove bubbles from all wells by gently squeezing a wash bottle containing 70-100% ethanol (with the inner straw removed) to blow vapor over the surface of each well.
- After placing the plate in the IncuCyte® live-cell analysis system, allow the plate to warm to 37 °C for 30 minutes prior to scanning.
- If contamination occurs within wells of the plate we recommend treating the affected wells with 1 M NaOH for 2-3 hours. Aspirate the well, taking care not to generate any aerosols, and leave the well empty for the remainder of the assay.

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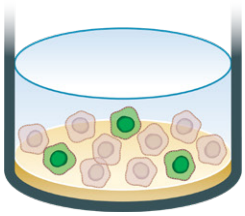
Angiogenesis PrimeKit assay protocol

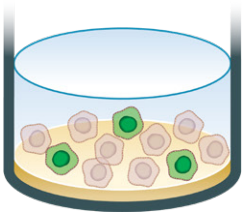
Quick Guide

1 SEED CELLS



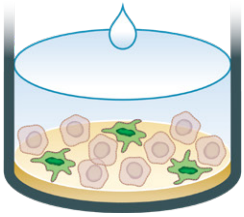
Seed NHDFs (100 µL/well) into a 96-well plate and allow to adhere for 1 hr. Seed GFP-HUVECs into the 96-well plate containing NHDFs and allow cells to adhere for 1 hr before placing them into the IncuCyte® system.

2 REPLACE MEDIUM



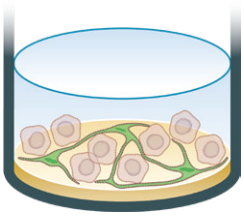
Carefully remove seeding media from wells containing cells and replace with complete growth medium (150 µL/well).

3 TREATMENT ADDITION



Prepare dilutions of test compounds in warmed complete assay medium. Carefully remove growth media from wells containing cells and replace with assay medium ± treatments (150 µL/well). Return plate to the IncuCyte system.

4 RE-FEED



On days 4 and 7 (optionally day 9) carefully remove media from wells and replace with 150 µL of assay medium ± treatment. Return plate to the IncuCyte system.

Day 1

1 **Seed cells**

1.1. Pre-equilibrate seeding medium (40 mL; green dot) and seeding media supplement (2 mL; green label) for 20-30 minutes in 37°C water bath. To make complete seeding medium, add 2 mL seeding media supplement to seeding medium and mix.

1.2. Thaw cryovial of NHDFs by gently swirling in a 37°C water bath until a sliver of ice remains. Add 1 mL of seeding medium to cryovial containing NHDFs and transfer cells and medium to a 15 mL conical tube containing 2 mL of seeding medium. Wash the cryovial with an additional 1 mL of seeding medium and transfer to the 15 mL conical tube containing cells. Centrifuge tube at 200 x g for 4 minutes.

1.3. Aspirate supernatant from the conical tube and resuspend the NHDF cell pellet in 12 mL of complete seeding medium.

1.4. Using a multi-channel pipette, seed cells (100 µL per well) into the 96-well bottom microplate. Let plate sit for 1 hour at ambient temperature in culture hood to allow NHDF cells to settle.

1.5. After 1 hour at ambient temperature, seed the GFP-HUVECs using the same procedure as used above for thawing the NHDFs.

1.6. Using a multi-channel pipette, seed HUVECs (100 µL per well) into the 96-well plate containing NHDFs. Let plate sit for 1 hour at ambient temperature in culture hood to allow HUVECs to settle.

1.7. Place the plate in the IncuCyte Live-Cell Analysis System and allow the plate to warm to 37°C for 30 minutes prior to scanning.

a. Objective: 4x or 10x
b. Channel Selection: Phase and Green
c. Scan Type: IncuCyte S3 (Standard), FLR (Tiled FOV), ZOOM (4x = Standard, 10x = Tiled FOV)
d. Scan Interval: every 6-12 hours

Day 2

2 **Replace medium**

2.1. Pre-equilibrate growth medium (20 mL; red dot) and growth media supplement (0.4 mL; red label) for 20-30 minutes in 37°C water bath. To make complete growth medium, add 0.4 mL growth media supplement to growth medium and mix.

2.2. Remove the 96-well plate containing cells from the IncuCyte system and carefully aspirate seeding medium from the wells using a multichannel vacuum manifold or an aspirating pipette.

2.3. Using a multi-channel pipette, add 150 µL of complete growth medium to each well of the 96-well plate.

2.4. Return the plate to the IncuCyte system and continue to monitor cells.

Day 3

3 Cell Treatment addition

NOTES: It is recommended that treatments be run using the following criteria; for ≤ 25% change, use an n=6 per treatment; for > 25% change use n-4 per treatment. Dissolve treatments in the complete growth medium wherever possible. If necessary, treatments may be dissolved in other solvents such as DMSO or ethanol, however, solvent concentration should not exceed 0.1% (v/v).

3.1. Pre-equilibrate assay medium (125 mL; blue dot) and assay media supplement (2.5 mL; blue label) for 20-30 minutes in 37°C water bath. To make complete assay medium, add 2.5 mL assay media supplement to assay medium and mix.

3.2. Prepare dilutions of test compounds in the warmed complete assay medium.

NOTE: Do not attempt to mix test compounds directly in the culture plate as this will damage the cell co-culture monolayer.

Recommendations:

3.2.1. **Controls**: Use 4-8 ng/mL VEGF to see maximal stimulation and 100 µM suramin in the presence of VEGF to see maximal inhibition.

3.2.2. **Pro-angiogenic treatments**: Add test treatments in the presence of low growth factors (1 ng/mL VEGF or bFGF gives a response that is 30-40% of maximum).

3.2.3. **Anti-angiogenic response treatments**: Add test treatments on Day 2 and at all subsequent feedings in the presence of 4-8 ng/mL VEGF.

3.2.4. **Vascular disruption**: Allow vascular networks to form in the presence of 4-8 ng/mL VEGF until at least Day 7 prior to the addition of the test treatments. At that point, introduce the test treatments in the maintained presence of VEGF, if desired.

3.2.5. **Conditioned media**: Conditioned medium from other cell cultures may be diluted in the fresh assay medium supplied with this kit and added directly to the angiogenesis plate. As a guideline, conditioned medium should be diluted at least 1:1 in fresh medium. This recommendation, however, must be optimized for each conditioned medium tested.

3.3. Remove the NHDF-HUVEC 96-well plate from the IncuCyte system and carefully aspirate growth medium from the wells using a multichannel vacuum manifold or an aspirating pipette.

3.4. Using a multi-channel pipette, add 150 µL of complete assay medium containing treatment to each well of the 96-well plate.

3.5. Return the plate to the IncuCyte system and continue to monitor cells.

Day 4

4 Re-feed assay plate

NOTE: If continuing assay to 10 days, additional feeding on Day 9 may be necessary.

4.1. Pre-equilibrate complete assay medium for 20-30 minutes in 37°C water bath and prepare required test treatment dilutions as described in step 3 above.

4.2. Removed the 96-well plate containing cells from the IncuCyte system and carefully aspirate assay medium from the wells using a multichannel vacuum manifold or an aspirating pipette.

4.3. Using a multi-channel pipette, add 150 µL of complete growth medium to each well of the 96-well flat plate.

4.4. Return the plate to the IncuCyte system and continue to monitor cells.

Image analysis

Vascular tube formation is quantified using the IncuCyte® Angiogenesis software module. Following imaging, the analysis parameters provided in this section will yield good starting values for identifying tube formation.

System	Threshold Parameters	Tube Identification Parameters		Filter Settings	
		Max Tube Width (µm)	Min Branch Length (µm)	Min Network Length (mm)	Min Tube Width Uniformity
• S3 • ZOOM	Use preset values. After preview, adjusting if necessary	100	50	0.2	0.6
• FLR	Background Intensity (AU): 8.0 (AU): 15.0 Manual Adjustment: 0.8	90	50	0.2	0.65

A complete suite of cell health applications is available to fit yourexperimental needs. Find more information at **essenbioscience.com/cellhealth** and **essenbioscience.com/immuno-oncology**

For additional product or technical information, please e-mail us at **AskAScientist@essenbio.com** visit our website at **essenbioscience.com** or call
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