Millicell® µ-Angiogenesis Inhibition Assay

Catalogue No. MMA125

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Not for use in diagnostic procedures

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Introduction

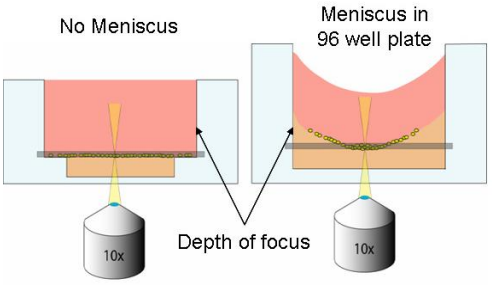
Angiogenesis, the formation of new blood vessels from a pre-existing vascular network, occurs normally in development and is critical for a majority of the vessel formation that occurs during embryogenesis, tissue generation, and wound healing. However, abnormal blood vessel growth, either excessive or insufficient, can be the underlying cause of many deadly and debilitating diseases including cancer, cardiovascular disease, stroke, and diabetic and age-related blindness. Identification of specific compounds that promote or inhibit angiogenesis may provide promising treatments for these diseases.

Millipore's Millicell µ-Angiogenesis Inhibition Assay provides an efficient system for the rapid and accurate identification of factors and inhibitors that reduce tube formation by endothelial cells. Included in the kit are the following components:

(1) Five µ-Angiogenesis Slides: Each chamber slide contains 15 wells for a total of 75 wells per kit. The µ-Angiogenesis Slides are based on the ibidi® technology platform, and possess enhanced optical imaging capabilities over the standard 96-well plate. The biocompatible plastic composition of the µ-Angiogenesis Slide provides an even, flat surface that eliminates the meniscus effect routinely observed in 96-well plates while providing similar high optical qualities observed in glass slides. As a result, all of the cells on the µ-Angiogenesis Slide are in focus; whereas, in a 96 well plate, only the few cells located at the center of the plate are in focus.



µ-Slide Angiogenesis Features	
Number of wells	15
Volume inner well	10 µL
Diameter inner well	4 mm
Depth inner well	0.8 mm
Volume upper well	50 µL
Diameter upper well	5 mm
Growth area inner well	0.12 cm^2
Coating area using 10 µL	0.23 cm^2



- All cells in focus
- 10µL gel per well
- No meniscus
- Excellent optics
- 15 wells
- Slide format

- Few cells in focus
- 100µL gel per well
- Meniscus formation
- Poor optics
- 96 wells
- Multi-well format

The µ-Angiogenesis Slide is compatible with multi-channel pipettes for easy filling and aspiration. It is also compatible with various fixation reagents such as methanol, paraformaldehyde, and other chemicals. Live cells can be observed directly or fixed and stained for further analysis.

(2) 1 vial of ECMatrix™ Gel Solution: A soluble basement membrane purified from Engelbreth Holm-Swarm (EHS) mouse tumor. It solidifies at room temperature to form a gelatinous substrate consisting of laminin, collagen type IV, heparin sulfate proteoglycans, entactin, and nidogen. Various factors (TGF-β, FGF) and proteolytic enzymes (plasminogen, tPA, MMPs) are also present in ECMatrix Gel Solution. ECMatrix Gel Solution is provided to capitalize upon the tendency of endothelial cells to form capillary-like structures upon exposure to the Gel Solution.

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(3) 1 vial of D, L-Sulforaphane: A naturally occurring compound found in cruciferous vegetables such as broccoli and cauliflower, it exhibits anti-cancer, anti-diabetic, and anti-microbial properties. Sulforaphane, a well-known agent that inhibits the tube formation ability of endothelial cells, is included as a useful control for inhibition studies.

(4) 1 vial of Calcein AM: A non-fluorescent, hydrophobic compound that can be readily transported across the membrane of intact, live cells. Calcein AM is provided as a simple, rapid, and accurate indicator of cell viability and cytotoxicity, and for short-term labeling of live cells. Once inside the cell, the acetoxymethyl (AM) ester group is hydrolyzed by intracellular esterases, yielding the free Calcein, a strongly fluorescent compound that is hydrophilic and thus well retained inside the cell cytoplasm. Apoptotic and dead cells are not labeled by Calcein AM, as they lack active esterases and also have compromised cell membranes which cannot retain the Calcein. Calcein is optimally excited at 495 nm and has a peak emission of 515 nm. For fluorescence microscopy, Calcein can be detected using filters for FITC.

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Kit Components

1. Millicell µ-Angiogenesis Slides: (Part No. CS203030) Five tissue culture treated slides. Each slide contains 15 wells. Store at room temperature.
2. ECMatrix Gel Solution: (Part No. 90060) One 1 mL vial. Store at -20 °C.
3. Calcein AM: (Part No. CS202541) One 50 µg vial. Store at -20 °C.
4. D, L-Sulforaphane: (Part No. CS203032) One 1 mg vial. Store at -20 °C.

Materials Required But Not Supplied

1. Inverted light microscope
2. Fluorescence microscope (if Calcein AM is used)
3. Precision pipettes
4. Accutase™ Cell Dissociation Solution (Cat. No. SCR005)
5. Human umbilical vein endothelial cells (HUVEC) (Cat. No. SCCE001) or any other experimental cell lines capable of tube formation
6. EndoGRO™ LS complete medium (Cat. No. SCME001) or other endothelial cell basal medium.
7. Dulbecco's Phosphate Buffered Saline (1X PBS) (Cat. No. BSS-1005-B)
8. EmbryoMax® ES Cell Qualified Ultra Pure Water, sterile H_2O (Cat. No. TMS-006)
9. Low speed centrifuge
10. Sterile microcentrifuge tubes
11. 37 °C incubator with 5% CO_2
12. Hemacytometer
13. Trypan Blue
14. DMSO

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Storage and Handling

1. Millicell µ-Angiogenesis Slides (Part No. CS203030) should be stored at room temperature until ready to use.
2. ECMatrix Gel Solution (Part No. 90060) should be stored at -20 °C until ready to use. Upon thawing, store at 2–8 °C for up to one week. Do not refreeze after thawing as this may affect efficacy.
3. Calcein AM (Part No. CS202541) is provided as 50 µg lyophilized powder. To prepare 1 mM Calcein AM Stock Solution, briefly centrifuge before opening tube and then add 50 µL sterile DMSO (not provided). Vortex vigorously. Centrifuge briefly to collect solution. Unused Calcein AM solution may be stored in aliquots at -20 °C for up to 1 month.
4. D, L-Sulforaphane (Part No. CS203032) is provided as 1 mg lyophilized powder. To prepare a 50 mM Stock Solution, dissolve powder in 112 µL sterile DMSO. Store reconstituted D, L-Sulforaphane solution at -20 °C for up to 3 months. Avoid repeated freeze/thaw cycles.

Assay Instruction

The following procedure is recommended for HUVEC cells and may be used as a reference point to further optimize specific cell type(s) of interest.

Day 0:

1. Culture HUVEC cells in a 10 cm tissue culture dish containing 10 mL EndoGRO LS Complete Medium (Cat. No. SCME001). Allow cells to grow until they reach 80–90% confluence.

   Note: *The EndoGRO LS Complete Medium contains 2% fetal bovine serum, 5 ng/mL EGF and other additional culture supplements. For optimal results, low passage (passages 1 through 8) HUVEC or other endothelial cells are recommended*.

2. Thaw ECMatrix Gel Solution in a 2–4 °C refrigerator overnight ("frost-free" refrigerators may generate temperatures above 4 °C and cause ECMatrix Gel Solution to gel). Keep ECMatrix Gel Solution vials on ice at all times.

3. Preparing and equilibrating the µ-Angiogenesis Slides:

   Because of the microscale size of the wells in the µ–Angiogenesis Slides, a humidified environment is recommended to prevent evaporation and to obtain optimal results. All procedures should be performed in a biological hood utilizing aseptic technique to prevent contamination.

   a. To create a humidified chamber, place two wet Kimwipes® wipers, folded in quarters, into a 10 cm Petri dish.
   b. Carefully open the µ-Angiogenesis Slide and remove the lid and slide from the packaging.
   c. Place the µ-Angiogenesis Slide in the humidified chamber (i.e., 10 cm Petri dish with wet Kimwipes wipers) and place the humidified chamber in a 37 °C, 5% CO_2 tissue culture incubator. *(Equilibrating the µ-Angiogenesis Slide inside the incubator overnight will help prevent air bubbles from becoming entrapped in the ECMatrix Gel in downstream steps.)*

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Day 1:

4. Transport the humidified chamber containing the µ-Angiogenesis Slide to the tissue culture hood.
5. Carefully and slowly aliquot 10 µL of ECMatrix Gel Solution that has been thawed at 2–8 °C or on ice overnight (from Step 2) into the inner well of each of the 15 wells of the µ-Angiogenesis Slide. To avoid generating air bubbles, it is best to aliquot slowly.

   Note: *ECMatrix Gel Solution should be kept on ice at all times. Use pre-cooled pipettes, plates, and tubes when preparing ECMatrix Gel Solution. ECMatrix Gel Solution is highly viscous and it may be necessary to cut off the end of the pipette tips with a sterile knife to facilitate ease of pipetting. Gelled ECMatrix Gel Solution may be re-liquified by placing at 0–4 °C for 24–48 hours, however efficacy may be affected.*
6. Place the lid over the µ-Angiogenesis Slide. Close the humidified chamber.
7. Visually inspect each well under a light microscope to ensure that the ECMatrix Gel Solution is evenly distributed across the well.
8. Carefully transport the humidified chamber containing the µ-Angiogenesis Slide to a 37 °C, 5% CO_2 tissue culture incubator. Incubate at 37 °C for one hour to allow the ECMatrix Gel Solution to solidify.

   Note: *The ECMatrix Gel-coated µ-Angiogenesis Slide should be used soon after polymerization. Prolonged polymerization will reduce tube formation.*
9. While the ECMatrix Gel Solution is solidifying, harvest the HUVEC or other endothelial cells.
   a. Carefully remove the medium from the 10 cm tissue culture dish containing 80–90% confluent HUVEC cells or other endothelial cells.
   b. Wash the cells once with 10 mL sterile 1X PBS. Aspirate the 1X PBS.
   c. Add 3 mL Accutase Cell Dissociation Solution (Cat. No. SCR005) and incubate in a 37 °C incubator for 5–15 minutes.
   d. Inspect the dish and ensure the complete detachment of cells by gently tapping the side of the dish with the palm of your hand.
   e. Apply 10 mL EndoGRO LS Complete Medium (pre-warmed to 37 °C) to the dish.
   f. Gently rotate the dish to mix the cell suspension. Transfer the dissociated cells to a 15 mL conical tube.
   g. Centrifuge the tube containing the cells at 1500 RPM for 5–10 minutes to pellet the cells.
   h. Aspirate and discard the supernatant. Be careful to not disturb the cell pellet.
   i. Apply 2 mL EndoGRO LS Complete Medium to the conical tube and resuspend the cells thoroughly. ***Note****: Do not vortex the cells.*
10. Count the number of cells using a hemacytometer. Adjust the cell concentration with additional EndoGRO LS Complete Medium to obtain the following:
    a. For wells containing the cell suspension alone without any test compounds or the inhibition control (sulforaphane) added, make up a 1–3 x 10^5 cells/mL cell suspension.
    b. For wells containing the cell suspension with the addition of test compounds (e.g., cytokines, pharmacological agents, etc.) or the inhibition control, sulforaphane, make up a 2–6 x 10^5 cells/mL cell suspension.

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Note: *It is recommended that at least one well of the µ-Angiogenesis Slide contain the inhibition control, sulforaphane, as a reference point for comparison.*

11. **Optional: Preparation of Sulforaphane as an inhibition control**
Because the total volume that the upper well of the µ-Angiogenesis Slide can accommodate is 50 µL, it is necessary to prepare the cell suspension and sulforaphane or other test compounds in 2X concentrations separately before mixing in equal parts to obtain a final 1X concentration of 50 µL final volume.
    a. Dissolved 1 mg sulforaphane with 112 µL DMSO to make a 50 mM stock solution.
    b. Further dilute the 50 mM Sulforaphane Stock solution to 100 µM Stock Solution by diluting 2 µL 50 mM Sulforaphane Stock Solution (from Step 11a) with 988 µL EndoGRO LS Complete Medium.
    c. Mix 25 µL of 100 µM Sulforaphane Stock Solution with 25 µL cell suspension (2–6 x 10^5 cells/mL from Step 10b) to make a total volume of 50 µL.

        Note: *For HUVEC cells, 50 µM sulforaphane is sufficient to inhibit angiogenesis. For other cell types, some optimization may be required to determine the maximal level of inhibition.*
12. Carefully add 50 µL of the appropriate cell suspension (from Steps 10a and 11c) into each of the upper wells of the µ-Angiogenesis Slide containing the polymerized ECMatrix Gel (from Step 8). This is equivalent to approximately 5,000–15,000 cells total per well. Do not touch the gel matrix with the pipette tip.
13. Incubate at 37 °C in a 5% CO_2 incubator.
14. The formation of tubes can be monitored either at fixed intervals, or in real time using an inverted light microscope connected to an environmental control chamber. Cellular network structures are fully developed by 12–18 hours, with the first visible signs apparent after 5–6 hours. After 24 hours, cells will begin to undergo apoptosis. Optimal times may vary depending on the cell type, cell age, and media growth conditions.
15. **Optional: Fluorescent monitoring of tube formation with Calcein AM staining**
    a. Prepare a 1 mM Calcein AM stock solution by dissolving 1 vial of 50 µg Calcein AM (provided) with 50 µL of DMSO.
    b. Vortex vigorously and centrifuge briefly to collect the solution.
    c. Dilute 1 µL of 1 mM Calcein AM stock solution with 19 µL EndoGRO LS Complete Medium to obtain a 50 µM Calcein AM stock solution.
    d. Add 1 µL of the 50 µM Calcein AM stock solution to each well of the µ-Angiogenesis Slide.
    e. Incubate at 37 °C for 15 minutes.
    f. Replace the staining solution with 50 µL EndoGRO LS Complete Medium.
    g. Observe the cells using a fluorescence microscope containing a FITC filter.

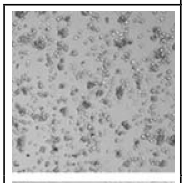
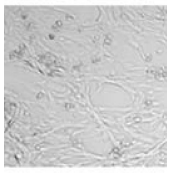
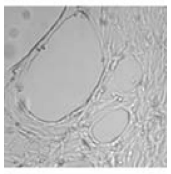
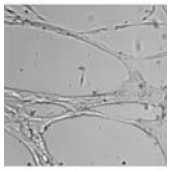
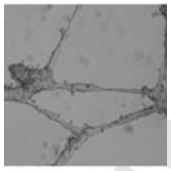
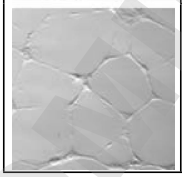
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Quantitation of Results

Activated endothelial cells form cellular networks (mesh-like structures) from capillary tubes sprouting into the stromal space (see Figures 1A and 2A). The formation of these cellular networks is a dynamic process, starting with cell migration and alignment, followed by the development of capillary tubes, sprouting of new capillaries, and finally the formation of the cellular networks. The following are guidelines to provide quantitative assessment of these cellular networks.

A. Pattern recognition

Define visual patterns by looking at or photographing the cells with 5X–20X objectives at set times at 37 °C after seeding on the ECMatrix Gel (end-point assay). Assign a numerical value to each pattern. This way a numerical value is associated with a degree of angiogenesis progression. An example is presented in the table below.

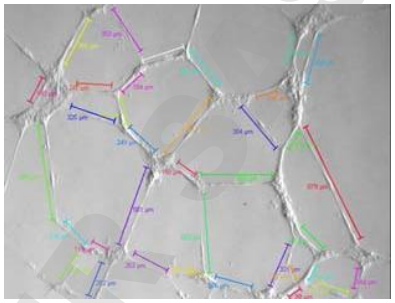
	Pattern	Value
	Individual cells, well separated	0
	Cells begin to migrate and align themselves	1
	Capillary tubes visible, no sprouting	2
	Sprouting of new capillary tubes visible	3
	Closed polygons begin to form	4
	Complex mesh-like structures develop	5

The pattern/value association criteria should be defined with the types of cells and experimental conditions that will be used in the angiogenesis assay. Several random fields-of-view (3–10) per well should be examined and the values averaged. This quantitation method will work best in assays involving potent inhibitors or activators of angiogenesis.

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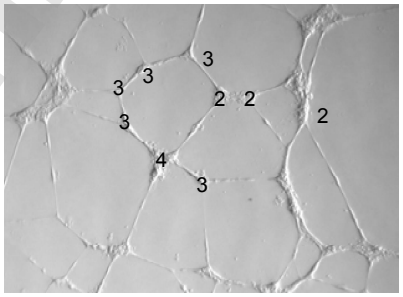
B. Branch Point Counting

A subtler, but more labor-intensive way to quantitate the progression of angiogenesis is to count the capillary tube branch points formed after a set amount of time (end-point assay). The length of the newly formed capillary tubes can also be taken into account when counting (do not count if shorter than an arbitrary predetermined length). Branch points in several random fields-of-view (3–10) per well should be counted and the values averaged.



C. Total Capillary Tube Length Measurement

An alternative method to branch point counting, suitable particularly for microscopes with imaging capabilities, is to measure the total length of all the capillary tubes in a field-of-view. The total capillary tube length in several random fields-of-view (3–10) per well should be examined and the values averaged.



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Results

The following figures are typical results obtained using HUVEC and HMVEC-d cells and should be used for reference purposes only.

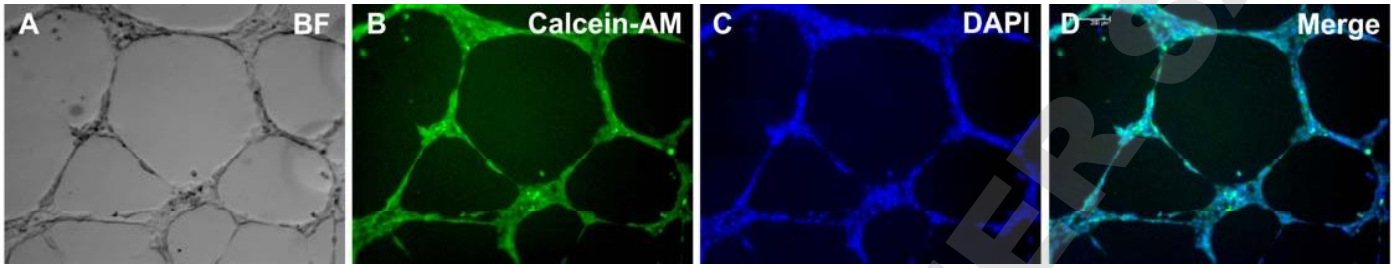


Figure 1. HUVEC cells form tubular structures on ECMatrix Gel. HUVEC cells (Cat. No. SCCE001) were cultured at 3 x 10^5 cells/mL per well on a µ-Angiogenesis Slide at passage 4 for 18 hours at 37 °C. Cells were then stained with 1 µM Calcein AM and 1 µg/mL DAPI for 15 minutes in culture media. Staining reagent was removed and replaced with regular growth medium before imaging. (**A**) Bright field image, (**B**) Green fluorescent Calcein AM staining, (**C**) Cell nuclei stained with DAPI and (**D**) Merged image of Calcein AM and DAPI.

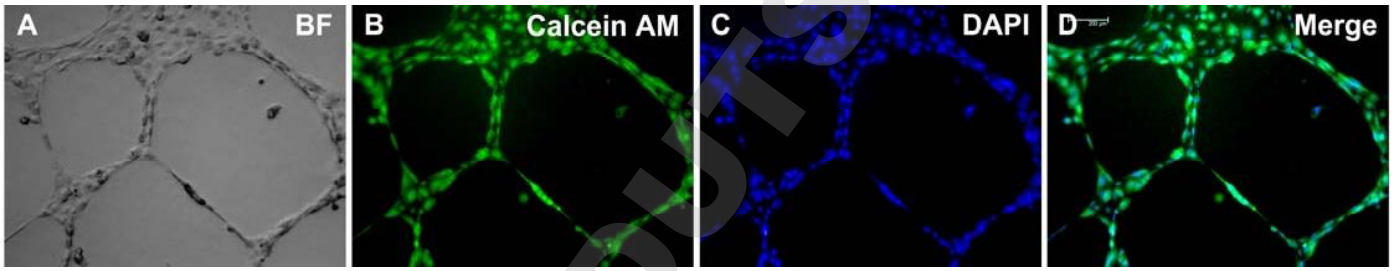


Figure 2. Human dermal microvascular endothelial (HMVEC-d) cells form tubes on ECMatrix Gel. HMVEC-d cells were cultured at 2.5 x 10^5 cells/mL per well on a µ-Angiogenesis Slide at passage 12 for 18 hours at 37 °C. Cells were stained with 1 µM Calcein AM and 1 µg/mL DAPI stain for 15 minutes in culture media. Stain media was replaced with regular growth medium before imaging. (**A**) Bright field image, (**B**) Green fluorescent Calcein AM staining, (**C**) Cell nuclei stained with DAPI and (**D**) Merged image of Calcein AM and DAPI.

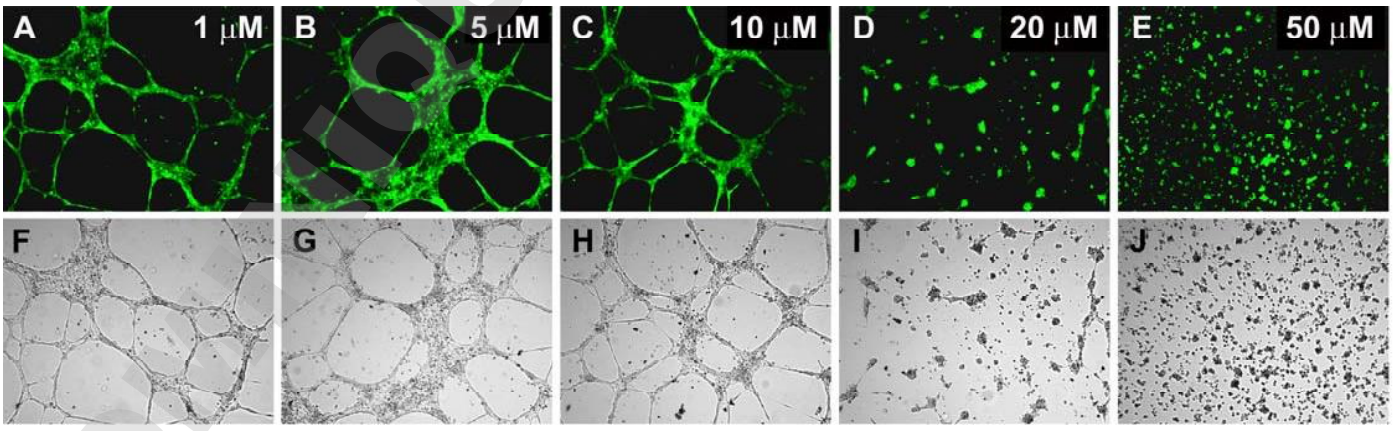


Figure 3. Sulforaphane inhibits tube formation of HUVEC cells. HUVEC cells were cultured in 3 x 10^5 cells/mL per well on a µ-Angiogenesis Slide at passage 6 for 18 hours in the presence of sulforaphane at the indicated concentrations. (**A – E**) Fluorescent images of cells stained with Calcein AM. (**F – J**) Bright-field images of sulforaphane treated cells.

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