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Primary Human Umbilical Vein Endothelial Cells

A Qualitative, Real-time, *In-vitro* Tube Formation Assay

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Angiogenesis – the formation of new blood vessels – is a process involving the migration, growth, and differentiation of endothelial cells that line the inside walls of blood vessels. Angiogenesis is a normal and vital process in growth and development, reproduction, wound healing, and the formation of granulation tissue[1-3]. It is also a fundamental process in the transition of tumors from benign to malignant states as well as invasion and metastasis[4,5], and plays a critical role in a variety of other diseases in which therapeutic treatment requires either the promotion of angiogenesis (e.g., limb ischemia[6]) or inhibition of angiogenesis (e.g., cancer or retinopathy[7]). Angiogenesis can be modeled *in vitro* through tube formation assays that visually track the migration and formation of endothelial cells into tubes, which are the basis of capillaries. These assays can be used to test the efficacy of compounds that either inhibit or promote angiogenesis. In this technical note, we demonstrate the successful development of a tube formation assay using Lonza's primary human umbilical vein endothelial cells (HUVECs) that can be used to visualize angiogenesis in real-time.

We tested four basement membrane substrates at three seeding densities and determined the most optimal combination for use with the Incucyte®. In the resulting assay, angiogenesis initiates within three hours and is demonstrably inhibited by the anti-angiogenic compound suramin. Furthermore, using the Incucyte®, real-time images can be obtained and analyzed at many time points without disrupting the assay, allowing for potential quantification of angiogenesis across a large variety of timepoints, as well as the development of time-lapse video.

Materials

Material used in this study for establishing the optimized tube formation assay are listed in Table 1.

Methods

Tube formation assays were prepared in 96-well tissue culture plates according to the detailed instructions found in the protocol published on the Lonza website[8]. The following steps briefly cover the protocol used in this study.

Culturing HUVECs

Lonza EGM® 2 Growth Media was prepared according to Lonza's recommended instructions[9]. Primary HUVEC cells (Lonza part no. C2519AS) were thawed (one amp at a time), counted with trypan blue and a hemocytometer, then plated at a density of 2,500 cells/cm^2 into T-25 flasks. Cells were incubated at 37°C and 5% CO_2 with media changes every 2 ± 1 days until reaching 70 – 90% confluence, at which point they were trypsinized and passaged into T-75 flasks using HEPES-BSS, trypsin and TNS (trypsin neutralizing solution). Cells were monitored and media changed every 2 ± 1 days until reaching 70 – 90% confluence for final passage at passage 2 into the 96-well tissue culture plates.

Plating HUVECs

Basement membrane matrices were prepared and plated according to manufacturer instructions for use in an angiogenesis application. We tested four matrices: one mouse-derived matrix (GIBCO® Geltrex™), 2 human-derived matrices (competitor A and competitor B), and one chemically-derived matrix (competitor C). Geltrex™ was thawed in a refrigerator one day prior to plating HUVECs and was added to plates followed by a 30 minute incubation at 37°C before plating HUVECs. Matrices from competitors A, B and C were prepared according to manufacturer instructions.

On Day 0 of the angiogenesis assay, HUVECs were harvested by trypsinization using HEPES-BSS, trypsin and TNS to detach cells. HUVECs were counted with trypan blue and a hemocytometer and then transferred to a conical tube at a density of 1×10^6 cells/mL. CellTracker® Dye was added according to the manufacturer's recommendations to a final concentration of 5 µM and incubated for 15 – 45 minutes, after which cells were diluted with medium and then centrifuged. Supernatant was then aspirated and replaced with media to bring cells to a density of 1×10^6 cells/mL, then plated into the tissue culture plates on top of each respective membrane matrix at densities of 12,000 – 50,000 cells per well. Either medium or medium combined with suramin was added to each well. Plates were placed in the Incucyte® in the incubator for up to 5 days at 37°C and 5% CO_2 for imaging.

Angiogenesis Assay

Angiogenesis was assessed qualitatively using the Incucyte®, ensuring that parameters were set to the brand of 96-well plate used (Corning® Falcon™) and analysis definition was optimized to the magnification being used. In this study, we chose a 20X magnification in order to better visualize the cells as they formed tubes; however, the Angiogenesis software module was not able to accurately quantify tube formation at this magnification. We recommend using either 4X or 10X magnification for quantitative assessments.

The overall study tested four types of membrane matrices at varied seeding densities for each matrix. As negative controls, we also tested all four membrane types using medium without growth supplements (EBM® 2 Basal Medium) and both types of media (EBM® 2 and EGM® 2) without a basement membrane to show that angiogenesis would not occur without all of the supporting elements. Once the optimum conditions for angiogenesis were determined, we used these conditions to test the effect of suramin at eight concentrations ranging from 0 µM to 780 µM. Three different lots of pooled HUVEC cells validated for angiogenesis markers (Lonza part no. C2519AS) were used to confirm the results.

Results and discussion

A previous study from 2017 at Lonza established a tube formation assay using primary HUVECs optimized for use with Corning® Matrigel®[10]. While that method is also an effective way to establish a tube formation assay, our study was optimized using Lonza HUVEC cells that have been validated to express 4 angiogenic markers (Lonza part no. C2519AS). Additionally, this study utilized real-time imaging using the Incucyte® and a different gel-based matrix (Geltrex™) that is optimized for angiogenesis.

Initial comparisons between the four gel types at different seeding densities showed that, when using Lonza HUVECs and EGM® 2 Medium, seeding 50,000 cells/well in a 96-well plate using Gibco® Geltrex™ as the membrane matrix resulted in excellent angiogenesis for all three lots (Figure 1) at 3 hours. These HUVEC cultures performed well up to 24-hours, at which point cell health had begun to decline, as expected with this matrix support. Geltrex™ performed the best using under the conditions and protocol we tested when compared to all 3 competitors (Figure 2A–C). The Incucyte® was unable to properly image wells using gels from competitor A or B (Figure 2A–B), which may have been due to the high concentration of the gel and/or the fact that manufacturer instructions require

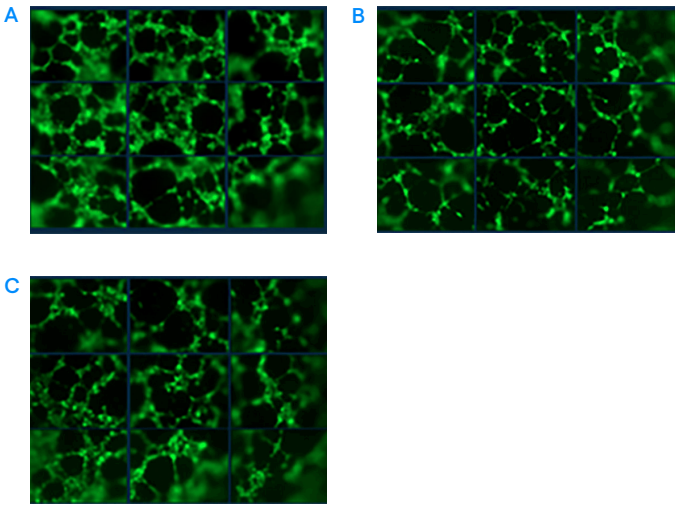

Figure 1.
Angiogenesis (tube formation) at 6 hours post-plating for select wells of HUVEC cells plated in EGM® 2 Medium at 50,000 cells/well on GIBCO® Geltrex™ membrane matrix. Lots used of Lonza HUVECs (part no. C2519AS) included (A) 22TL073029, (B) 22TL164356, and (C) 22TL199777. Images are comprised of 9 different positions within the same well.

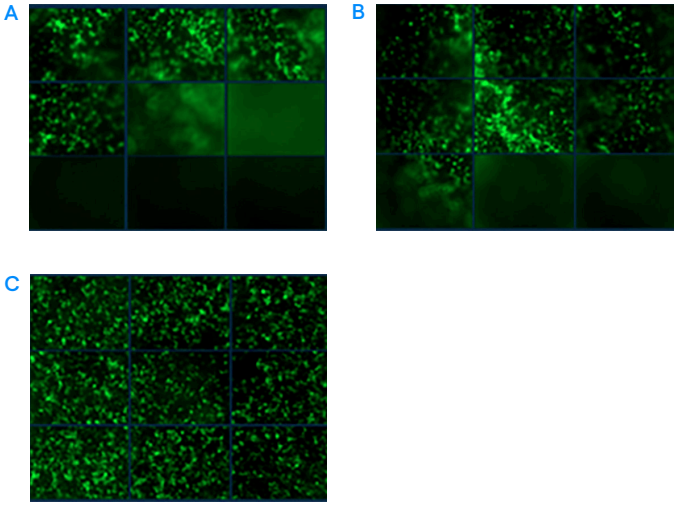

Figure 2.
Representative images of wells plated with lot 22TL073029 of HUVECs (part no. C2519AS) in EGM® 2 Medium using the following support matrices and cell densities: (A) competitor A at 30,000 cells/well, 48-hours post overlay (B) competitor B at 30,000 cells/well, 48-hours post overlay, and (C) competitor C at 50,000 cells/well at 6 hours post-plating . Note the lack of tubule formation in any of the images. Concentrations were titrated based on manufacturer's recommendations. Other cell densities were also unsuccessful (data not shown). Both matrices from competitors A and B interfered with the Incucyte's® ability to focus. Images are comprised of 9 different positions within the same well.

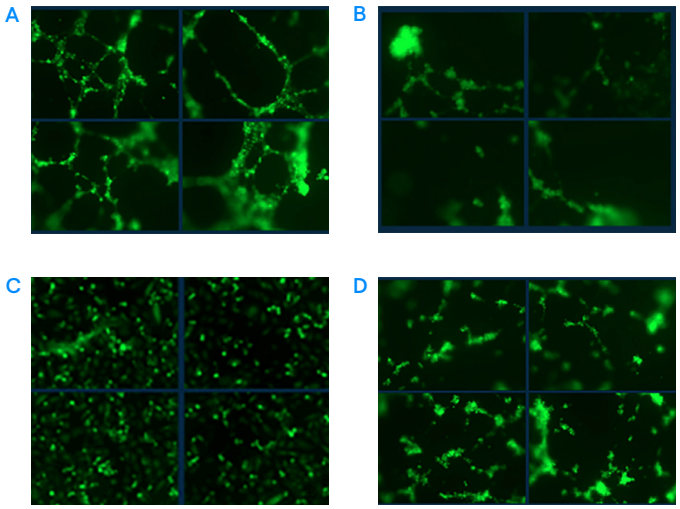

Figure 3.
Representative images of suramin inhibition in angiogenesis assay using lot 22TL073029 of HUVECs (part no. C2519AS) plated for 6 hours with the following conditions: (A) HUVECs + Geltrex™ + EGM® 2 + a non-inhibitory dose of 0.78 µM suramin. (B) HUVECs + Geltrex™ + EGM® 2 + an inhibitory dose of 24.5 µM suramin. Generally, above 20 µM is considered an inhibitory dose. Also shown are controls (C) HUVECs + no gel + EGM® 2 + a non-inhibitory dose of 0.78 µM suramin and D) HUVECs + Geltrex™ + EBM® 2 Basal Medium + a non-inhibitory dose of 0.78 µM suramin. Images are comprised of 4 different positions within the same well.

overlaying the matrix atop the cells. Further examination under light microscopy revealed small amounts of angiogenesis at the periphery of wells for these two competitor matrices, but the majority of the wells were obscured by the membrane matrix overlay. Additionally, competitor C did not result in angiogenesis under any of the conditions tested. As a result, further studies were performed using the Geltrex™ membrane matrix.

We ran an angiogenesis inhibition assay using an eight-point titration of suramin on all three donors plated at 50,000 cells/well using the Geltrex™ membrane matrix. The titrated concentrations ranged from 0–780 µM, with >20 µM generally considered to be an inhibitory dose[11]. Our results show that angiogenesis was clearly inhibited at and above 24.5 µM concentrations, but not below (Figure 3A–B). Controls without Geltrex™ or without supplemented medium showed no angiogenesis even when treated with non-inhibitory doses of suramin (Figure 3C–D). This demonstrates that the assay works as anticipated, qualitatively matching the dose response expected with suramin. We were also able to image multiple time points without disturbing the assay, allowing for the creation of time-lapse video that show the migration of cells and formation of tubes under the optimized assay conditions.

Using the Incucyte® Angiogenesis software module, it is possible to calculate a dose-response curve and an IC50 value based on the acquired imagery; however, we believe that the higher magnification of 20X used in this study challenged the software in properly distinguishing tubes from random clustering at that level. For full quantitation of angiogenesis, we recommend imaging at 4X or 10X

zoom to provide the software with a much larger number of data points to work with, thus eliminating issues caused by random variation in cell location.

Conclusions

We have developed an optimized, high-throughput angiogenesis tube formation assay utilizing Lonza HUVECs plated at 50,000 cells/well in a 96-well plate and Gibco® Geltrex™ as a basement membrane matrix. With a quick initiation of tube formation at 3 hours or less and full formation of tubes within 24-hours, this assay is robust, and therefore can allow for a quick turnaround time to assess compounds that may stimulate or inhibit angiogenesis. This assay can be used in conjunction with the Sartorius Incucyte® SX5 Live Cell Imager to generate both time-lapse video as well as quantitative measures of angiogenesis. This assay demonstrated the expected inhibitory dose response when exposed to a range of suramin concentrations, validating its use for drug development applications.

Vendor	Catalog no.	Description
Lonza	C2519AS	Human Umbilical Vein Endothelial Cells, Angiogenesis Qualified, ≥500,000 cells per amp
Lonza	CC-5012	Trypsin/EDTA, 100 mL bottle
Lonza	CC-5002	Trypsin Neutralizing Solution (TNS), 100 mL bottle
Lonza	CC-5024	HEPES Buffered Saline Solution (HEPES-BSS), 500 mL bottle
Lonza	CC-3156	EBM® 2 Basal Medium, 500 mL bottle
Lonza	CC-4176	EGM® 2 SingleQuots® Supplement Kit
ThermoFisher	A4000046701	GIBCO® Geltrex™ LDEV free reduced growth factor basement membrane matrix
ThermoFisher	C2925	CellTracker® Dye
Corning	3596	Corning® Falcon™ 96-well plates
Sartorius		Sartorius Incucyte® SX5 Live Cell Analysis Instrument
Sartorius	9600-0011	Incucyte® Angiogenesis Software Module
SigmaAldritch	S2671-100MG	Suramin sodium salt, 100 mg
Any		DMSO
Any		PBS
Any		Trypan Blue
Any		T25 tissue culture flasks
Any		T75 tissue culture flasks
Any		50 mL conical tubes

Table 1.
Materials used by Lonza to create the optimized HUVEC tube formation assay.

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